

LIPOXYGENASE PROTEINS AND NUCLEIC ACIDS

AN APPLICATION FOR  
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### Description

## LIPOXYGENASE PROTEINS AND NUCLEIC ACIDS

### Technical Field

The present invention relates generally to isolated  
5 and purified lipoxygenase proteins and nucleic acids.  
More particularly, the present invention relates to an  
isolated and purified second type of human 15S-  
lipoxygenase and an isolated and purified nucleic acid  
encoding the same, and to an isolated and purified nucleic  
10 acid encoding a mouse 8S-lipoxygenase.

### Table of Abbreviations

15-Lox-1	Reticulocyte-type of 15S-lipoxygenase
15-Lox-2	Second type of human 15S-lipoxygenase
8-Lox	mouse 8S-lipoxygenase
15 PMA	Phorbol-12-myristate-13-acetate
H(P)ETE	Hydro(pero)xyeicosatetraenoic acid
HODE	Hydroxyoctadecadienoic acid
HPLC	High pressure liquid chromatography
PCR	Polymerase chain reaction

RACE

Rapid amplification of cDNA ends

Grant Statement

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Background Art

The lipoxygenases are a structurally related family of non-heme iron dioxygenases that function in the production of fatty acid hydroperoxides. Three lipoxygenases have been identified and cloned in humans. Funk, C. D. (1993) *Prog. Nuc. Acid Res. Mol. Biol.* 45:67-98; Matsumoto et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:26-30; Dixon et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:416-420; Funk et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:5638-5642; Izumi et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:7477-7481; Yoshimoto et al. (1990) *Biochem. Biophys. Res. Comm.* 172:1230-1235; Sigal et al. (1988) *Biochem. Biophys. Res. Comm.* 157:457-464). They oxygenate arachidonic acid in different positions along the carbon chain and form the corresponding 5S-, 12S- or 15S-

hydroperoxides (hydroperoxy-eicosatetraenoic acids, HPETEs). The three enzymes are known mainly from the blood cell types in which they are strongly expressed - the 5S-lipoxygenase of leukocytes, the 12S-lipoxygenase of platelets, and the 15S-lipoxygenase of reticulocytes, eosinophils and macrophages. While these are the most widely recognized cellular sources, selective expression is well documented in other tissues. For example, both the 12S- and 15S-lipoxygenases are detected in skin.

10 Nugteren et al. (1987) *Biochim. Biophys. Acta* 921:135-141; Henneicke-von Zepelin et al. (1991) *J. Invest. Dermatol.* 97:291-297; Takahashi et al. (1993) *J. Biol. Chem.* 268:16443-16448; Hussain et al. (1994) *Amer. J. Physiol.* 266:C243-C253.

15 Potentially, the three cloned lipoxygenases could account for all enzymatic synthesis of arachidonate hydroperoxides in humans, but there are reasons to consider that other lipoxygenases may exist. For example, in the mouse there are five known lipoxygenases, three that correspond to the known human enzymes, Chen et al. (1994) *J. Biol. Chem.* 269:13979-13987; Chen et al. (1995) *J. Biol. Chem.* 270:17993-17999 and two others, Furstenberger et al. (1991) *J. Biol. Chem.* 266:15738-15745; Funk et al. (1996) *J. Biol. Chem.* 271:23338-23344.

20

Three of the five distinct mouse lipoxygenase enzymes are best known for their occurrence in different types of blood cells. In common with other mammals, a 5S-lipoxygenase is present in leukocytes and is responsible for synthesis of the pro-inflammatory mediators, the leukotrienes. Chen et al. (1995) *J. Biol. Chem.* 270:17993-17999; Chen et al. (1994) *Nature* 372:179-182. A 12S-lipoxygenase is found in platelets and several other tissues including skin. Nugteren et al. (1987) *Biochim. Biophys. Acta* 921:135-141; Chen et al. (1994) *J. Biol. Chem.* 269:13979-13987; Sun et al. (1996) *J. Biol. Chem.* 271:24055-24062.

A second type of 12S-lipoxygenase which is closely related in sequence to the human and rabbit "reticulocyte-type" of 15S-lipoxygenases occurs in certain macrophages. Sun et al. (1996) *J. Biol. Chem.* 271, 24055-24062. The fourth mouse lipoxygenase to be characterized is another enzyme to have 12S-lipoxygenase activity; it was cloned recently from mouse skin and has been classified as an epidermal 12S-lipoxygenase. van Dijk et al. (1995) *Biochim. Biophys. Acta* 1259:4-8; Funk et al. (1996) *J. Biol. Chem.* 271:23338-23344. All four of these murine lipoxygenases enzymes have been characterized at the cDNA and genomic levels.

The fifth known mouse lipoxygenase was described originally in 1986 by Fürstenberger, Marks and colleagues as an enzyme in skin forming 8-HETE and inducible by phorbol ester treatment. Gschwendt et al. (1986) *Carcinogenesis* 7:449-455. It was shown subsequently that this enzyme forms the 8S enantiomer (Hughes et al. (1991) *Biochim. Biophys. Acta* 1081:347-354) and isolation of the corresponding hydroperoxide confirmed identification of the enzyme as a lipoxygenase. Fürstenberger et al. (1991) *J. Biol. Chem.* 266:15738-15745. Mouse skin is the only reported site of synthesis of 8S-HETE in animal tissues, and there is no indication from the literature pointing to a potential homologue of the mouse 8S-lipoxygenase in other mammals. Additionally, no nucleic acid, particularly a cDNA, which encodes this lipoxygenase has been characterized.

Despite the description in the art of the enzymes presented above, along with the catalytic activities covered by these enzymes, there remains an open question whether a lipoxygenase rather than a cytochrome P450 might account for the synthesis of 12R-hydroxy arachidonic acid (12R-HETE), Hammarström et al. (1975) *Proc. Natl. Acad. Sci. USA* 72:5130-5134; Woollard, P. M. (1986) *Biochem. Biophys. Res. Commun.* 136(1):169-175; Baer et al. (1991) *J. Lipid Research* 32:341-347; Holtzman et al. (1989) *J.*

Clin. Invest. 84:1446-1453; Brash et al. (1996) *J. Biol. Chem.* 271:20549-20557, a prominent arachidonate metabolite in the skin disease of psoriasis and other proliferative dermatoses (Hammarström et al. (1975) *Proc. Natl. Acad. Sci. USA* 72:5130-5134; Baer et al. (1991) *J. Lipid Research* 32:341-347; Baer et al. (1995) *J. Invest. Dermatol.* 104:251-255).

Therefore, what is needed, then, is further characterization of lipoxygenase enzymes in vertebrates, particularly in mammals, and more particularly in humans. A novel isolated and purified lipoxygenase and a nucleic acid encoding the same would have broad utility to due its role in arachidonic acid metabolism, a critical metabolic pathway.

#### Disclosure of the Invention

A key aspect of this invention pertains to the discovery of a novel 15S-lipoxygenase (15-Lox-2) protein and nucleic acid encoding the 15-Lox-2 protein. Preferred nucleic acid and amino acid sequences for 15-Lox-2 are described in SEQ ID NO:1 and SEQ ID NO:2.

It is another aspect of this invention that the novel 15-Lox-2 protein acts in the metabolism of arachidonic acid to 15S-Hydro(pero)xyeicosatetraenoic acid.

Another key aspect of this invention is isolation and purification of a nucleic acid encoding mouse 8S-

lipoxygenase (8-Lox). A preferred embodiment of this nucleic acid is described in SEQ ID NO:3.

Thus, in one aspect, the present invention provides an isolated and purified polynucleotide that encodes a  
5 lipoxygenase polypeptide wherein the lipoxygenase polypeptide includes an iron ligand comprising a serine. Preferably, the lipoxygenase polypeptide reacts with arachidonic acid. In a preferred embodiment, a polynucleotide of the present invention is a DNA molecule  
10 from a vertebrate species. A preferred vertebrate is a mammal. A preferred mammal is a human. More preferably, a polynucleotide of the present invention encodes polypeptides designated 15-Lox-2 and 8-Lox. Even more preferred, a polynucleotide of the present invention  
15 encodes a polypeptide comprising the amino acid residue sequence of SEQ ID NO:2 or SEQ ID NO:4. Most preferably, an isolated and purified polynucleotide of the invention comprises the nucleotide base sequences of SEQ ID NO:1 or SEQ ID NO:3 or their homologues from other vertebrate  
20 species.

Yet another aspect of the present invention contemplates an isolated and purified polynucleotide comprising a base sequence that is identical or complementary to a segment of at least 10 contiguous bases  
25 of SEQ ID NO:1 wherein the polynucleotide hybridizes to a

polynucleotide that encodes a lipoxxygenase polypeptide wherein the lipoxxygenase polypeptide includes an iron ligand comprising a serine. Preferably, the lipoxxygenase polypeptide reacts with arachidonic acid. Preferably, the  
5 isolated and purified polynucleotide comprises a base sequence that is identical or complementary to a segment of at least 25 to 70 contiguous bases of SEQ ID NO:1. For example, a polynucleotide of the invention can comprise a segment of bases identical or complementary to 40 or 55  
10 contiguous bases of the disclosed nucleotide sequences.

In another embodiment, the present invention contemplates an isolated and purified lipoxxygenase polypeptide wherein the lipoxxygenase polypeptide includes an iron ligand comprising a serine. Preferably, the  
15 lipoxxygenase polypeptide reacts with arachidonic acid. More preferably, a polypeptide of the invention is a recombinant polypeptide. Even more preferably, a polypeptide of the present invention is 15-Lox-2. Even more preferably, a polypeptide of the present invention  
20 comprises the amino acid residue sequence of SEQ ID NO:2.

In an alternative embodiment, the present invention provides an expression vector comprising a polynucleotide that encodes a lipoxxygenase polypeptide that includes an iron ligand comprising a serine. Preferably, the  
25 lipoxxygenase polypeptide reacts with arachidonic acid.

Also preferably, an expression vector of the present invention comprises a polynucleotide that encodes 15-Lox-2 or 8-Lox. More preferably, an expression vector of the present invention comprises a polynucleotide that encodes  
5 a polypeptide comprising the amino acid residue sequence of SEQ ID NO:2 or SEQ ID NO:4. More preferably, an expression vector of the present invention comprises a polynucleotide comprising the nucleotide base sequence of SEQ ID NO:1 or SEQ ID NO:3. Even more preferably, an  
10 expression vector of the invention comprises a polynucleotide operatively linked to an enhancer-promoter. More preferably still, an expression vector of the invention comprises a polynucleotide operatively linked to a prokaryotic promoter. Alternatively, an expression  
15 vector of the present invention comprises a polynucleotide operatively linked to an enhancer-promoter that is a eukaryotic promoter, and the expression vector further comprises a polyadenylation signal that is positioned 3' of the carboxy-terminal amino acid and within a  
20 transcriptional unit of the encoded polypeptide.

In yet another embodiment, the present invention provides a recombinant host cell transfected with a polynucleotide that encodes a lipxygenase polypeptide which includes an iron ligand comprising a serine.  
25 Preferably, the lipxygenase polypeptide reacts with

arachidonic acid. SEQ ID NO:1; SEQ ID NO: 2 SEQ ID NO:3;  
and SEQ ID NO: 4 set forth nucleotide and amino acid  
sequences from the exemplary vertebrates human and mouse.  
Also contemplated by the present invention are homologous  
5 or biologically equivalent polynucleotides and  
lipxygenase polypeptides found in other vertebrates.  
Preferably, a recombinant host cell of the present  
invention is transfected with the polynucleotide that  
encodes 15-Lox-2 or 8-Lox. More preferably, a recombinant  
10 host cell of the present invention is transfected with the  
polynucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3.  
Even more preferably, a host cell of the invention is a  
eukaryotic host cell. Still more preferably, a  
recombinant host cell of the present invention is a  
15 vertebrate cell. Preferably, a recombinant host cell of  
the invention is a mammalian cell.

In another aspect, a recombinant host cell of the  
present invention is a prokaryotic host cell. Preferably,  
a recombinant host cell of the invention is a bacterial  
20 cell, preferably a strain of *Escherichia coli*. More  
preferably, a recombinant host cell comprises a  
polynucleotide under the transcriptional control of  
regulatory signals functional in the recombinant host  
cell, wherein the regulatory signals appropriately control  
25 expression of a lipxygenase polypeptide that metabolizes

arachidonic acid in a manner to enable all necessary transcriptional and post-transcriptional modification.

In yet another embodiment, the present invention contemplates a process of preparing a lipxygenase polypeptide comprising transfecting a cell with polynucleotide that encodes a lipxygenase polypeptide which includes an iron ligand comprising a serine, to produce a transformed host cell; and maintaining the transformed host cell under biological conditions sufficient for expression of the polypeptide. Preferably, the lipxygenase polypeptide that is produced reacts with arachidonic acid. More preferably, the transformed host cell is a eukaryotic cell. More preferably still, the eukaryotic cell is a vertebrate cell. Alternatively, the host cell is a prokaryotic cell. More preferably, the prokaryotic cell is a bacterial cell of the DH5 $\alpha$  strain of *Escherichia coli*. Even more preferably, a polynucleotide transfected into the transformed cell comprises the nucleotide base sequence of SEQ ID NO:1 or SEQ ID NO:3. SEQ ID NO:1; SEQ ID NO:2; SEQ ID NO:3; and SEQ ID NO:4 set forth nucleotide and amino acid sequences for the exemplary vertebrates human and mouse. Also contemplated by the present invention are homologues or biologically equivalent lipxygenase polynucleotides and polypeptides found in other vertebrates.

In still another embodiment, the present invention provides an antibody immunoreactive with a lipoxxygenase polypeptide which includes an iron ligand comprising a serine. Preferably, the lipoxxygenase polypeptide reacts with arachidonic acid. SEQ ID NO:1; SEQ ID NO:2; SEQ ID NO:3; and SEQ ID NO:4 set forth nucleotide and amino acid sequences from the exemplary vertebrates human and mouse. Also contemplated by the present invention are antibodies immunoreactive with homologues or biologically equivalent lipoxxygenase polynucleotides and polypeptides found in other vertebrates. Preferably, an antibody of the invention is a monoclonal antibody. More preferably, the lipoxxygenase polypeptide comprises 15-Lox-2 or 8-Lox. Even more preferably, a polypeptide comprises the amino acid residue sequence of SEQ ID NO:2 or SEQ ID NO:4.

In another aspect, the present invention contemplates a process of producing an antibody immunoreactive with a lipoxxygenase polypeptide which includes an iron ligand comprising a serine, the process comprising the steps of (a) transfecting a recombinant host cell with a polynucleotide that encodes a lipoxxygenase polypeptide which includes an iron ligand comprising a serine; (b) culturing the host cell under conditions sufficient for expression of the polypeptide; (c) recovering the polypeptide; and (d) preparing the antibody to the

polypeptide. Preferably, the lipoxxygenase polypeptide reacts with arachidonic acid. SEQ ID NO:1; SEQ ID NO:2; SEQ ID NO:3; and SEQ ID NO:4 set forth nucleotide and amino acid sequences from the exemplary vertebrates mouse  
5 and human. Preferably, the host cell is transfected with the polynucleotide of SEQ ID NO:1 or SEQ ID NO:3. Even more preferably, the present invention provides an antibody prepared according to the process described above. Also contemplated by the present invention is the  
10 use of homologues or biologically equivalent polynucleotides and polypeptides found in other vertebrates to produce antibodies.

Alternatively, the present invention provides a process of detecting a lipoxxygenase polypeptide that  
15 metabolizes arachidonic acid, wherein the process comprises immunoreacting the polypeptide with an antibody prepared according to the process described above to form an antibody-polypeptide conjugate, and detecting the conjugate.

20 In yet another embodiment, the present invention contemplates a process of detecting a messenger RNA transcript that encodes a lipoxxygenase polypeptide which includes an iron ligand comprising a serine, wherein the process comprises hybridizing the messenger RNA transcript  
25 with a polynucleotide sequence that encodes that

polypeptide to form a duplex; and detecting the duplex. Alternatively, the present invention provides a process of detecting a DNA molecule that encodes a lipoxxygenase polypeptide, wherein the process comprises hybridizing DNA  
5 molecules with a polynucleotide that encodes a lipoxxygenase polypeptide which includes an iron ligand comprising a serine to form a duplex; and detecting the duplex. For both such processes, it is preferred that the detected lipoxxygenase polypeptide is capable of reacting  
10 with arachidonic acid.

In another aspect, the present invention contemplates a diagnostic assay kit for detecting the presence of a lipoxxygenase polypeptide in a biological sample, where the kit comprises a first container containing a first  
15 antibody capable of immunoreacting with a lipoxxygenase polypeptide which includes an iron ligand comprising a serine, with the first antibody present in an amount sufficient to perform at least one assay. Preferably, an assay kit of the invention further comprises a second  
20 container containing a second antibody that immunoreacts with the first antibody. More preferably, the antibodies used in an assay kit of the present invention are monoclonal antibodies. Even more preferably, the first antibody is affixed to a solid support. More preferably  
25 still, the first and second antibodies comprise an

indicator, and, preferably, the indicator is a radioactive label or an enzyme.

In an alternative aspect, the present invention provides a diagnostic assay kit for detecting the presence, in biological samples, of a lipoxxygenase polypeptide, the kits comprising a first container that contains a second polynucleotide identical or complementary to a segment of at least 10 contiguous nucleotide bases of a polynucleotide that encodes a lipoxxygenase polypeptide which includes an iron ligand comprising a serine. Preferably, the polynucleotide encodes a lipoxxygenase polypeptide capable of reacting with arachidonic acid. More preferably, the polynucleotide encodes 15-Lox-2 or 8-Lox.

In another embodiment, the present invention contemplates a diagnostic assay kit for detecting the presence, in a biological sample, of an antibody immunoreactive with a lipoxxygenase polypeptide, the kit comprising a first container containing a lipoxxygenase polypeptide which includes an iron ligand comprising a serine that immunoreacts with the antibody, with the polypeptide present in an amount sufficient to perform at least one assay. Preferably, the lipoxxygenase polypeptide is capable of reacting with arachidonic acid. More preferably, the polypeptide comprises 15-Lox-2 or 8-Lox.

The foregoing aspects and embodiments have broad utility given the biological significance of the arachidonic acid pathway, as is known in the art. By way of example, the foregoing aspects and embodiments are  
5 useful in the preparation of screening assays and assay kits that are used to identify compounds that affect arachidonic acid metabolism, or that are used to detect the presence of the proteins and nucleic acids of this invention in biological samples. Additionally, it is well  
10 known that isolated and purified polypeptides have utility as feed additives for livestock and further polynucleotides encoding the polypeptides are thus useful in producing the polypeptides.

Following long-standing patent law convention, the  
15 terms "a" and "an" mean "one or more" when used in this application, including the claims.

Some of the aspects and objects of the invention having been stated hereinabove, other aspects and objects will become evident as the description proceeds, when  
20 taken in connection with the accompanying drawings as best described hereinbelow.

#### Brief Description of the Drawings

Fig. 1 shows sequence alignment of human 15S-lipoxygenases. The top line shows the amino acid sequence

(SEQ ID NO:2) deduced from the new human lipoxxygenase (15-  
Lox-2). cDNA, in alignment with the sequence of the  
previously reported human 15S-lipoxxygenase (15-Lox-1) (SEQ  
ID NO:25). Sigal et al. (1988) *Biochem. Biophys. Res.*  
5 *Comm.* 157: 457-464. The consensus sequences used in PCR  
cloning are underlined, and five putative iron ligands  
(H374, H379, H554, S558, I676 - 15-Lox-2; H374, H379,  
H554, H558, I676 - 15-Lox-1) are in bold. Two clones of  
the new cDNA were sequenced: there was a single nucleotide  
10 difference (position 1263 in the open reading frame, C or  
T) which did not change the deduced amino acid sequence.  
The new cDNA sequence (SEQ ID NO:1) is available in the  
GenBank™/EMBL Data Bank with accession number U78294.

Fig. 2 shows expression in HEK 293 cells:  
15 identification of the 15S-HETE product. Following  
transient expression of the cDNA, the HEK 293 cells were  
sonicated in 50 mM Tris (pH 7.5) containing 100 mM NaCl,  
then incubated with [<sup>14</sup>C]arachidonic acid (50  $\mu$ M) for 30  
min at 37°C, and the products extracted as described IN  
20 Brash et al. (1996) *J. Biol. Chem.* 271:20549-20557.

Fig. 2A shows reversed-phase HPLC analysis of the  
products using a Beckman 5 $\mu$  ODS Ultrasphere column (25 x  
0.46 cm) with a Bio-Rad 5S ODS guard column, a solvent  
system of methanol:water:glacial acetic acid (80:20:0.01,  
25 by volume), and a flow rate of 1.1 ml/min with on-line

detection of radiolabeled products using a Packard Flo-One Radiomatic detector. Retention times of HETE standards are indicated on chromatogram. The small peak on the front shoulder of the 15-HETE is 15-keto-eicosatetraenoic acid.

5        Fig. 2B shows chiral analysis of the methyl ester derivative of the 15-HETE product using a Chiralcel OB column with a solvent of hexane:isopropanol (100:2, v/v) and a flow rate of 1.1 ml/min.

10        Figs. 3A and 3B shows multiple human tissue RNA blots. Two tissue blots of mRNA (Clontech) were probed with a 1067 bp fragment of the new human lipooxygenase cDNA.

Fig. 4 shows detection of the new 15S-lipooxygenase transcript in human cornea.

15        Fig.4A depicts RT-PCR wherein RNA samples were treated with DNase 1, then reverse transcribed to cDNA. PCR reactions were run using two primer sets (1 and 2, see Experimental Procedures of Example 1) with human cornea cDNA as template, and also with rabbit cornea cDNA and  
20        buffer (H<sub>2</sub>O) alone as negative controls. Bands of the correct sizes, 589 bp and 351 bp, are detected in human cornea; the larger band was subcloned and sequenced, confirming the identity to the lipooxygenase cDNA cloned from skin.

Fig. 4B depicts a Northern analysis of human eye tissues. The band in cornea mRNA at about 2.5-3 KB corresponds to the new lipoxxygenase transcript.

Figure 5 shows nucleotide (SEQ ID NO:3) and deduced amino acid (SEQ ID NO:4) sequences of the mouse 8S lipoxxygenase. The sequence is from two identical cDNA library clones, except for the 5' UTR, which was obtained by 5' RACE. The consensus sequences used in PCR cloning are underlined. Seven PCR clones encoding the open reading frame and which expressed 8S-lipoxxygenase activity were also fully sequenced. These contained multiple nucleotide substitutions which changed the encoded amino acid sequence yet had no apparent detrimental effect on their expressed 8S-lipoxxygenase activity: clone #G2, 112A (Leu to Met), 227-C (Val to Ala), 1607-A (Arg to Gln); clone #G5, 227-C (Val to Ala), 1607-A (Arg to Gln); clone #G11, 227-C (Val to Ala), 1607-A (Arg to Gln); clone #K1, 227-C (Val to Ala); clone #K2, same amino acid sequence as library clone; clone #K7, 1237-G (Ile to Val); clone #K12, 95-G (Glu to Gly), 173-G (Pro to Arg). The cDNA sequence is available in GenBank with accession no. U93277.

Figure 6 shows alignment of mouse 8S-lipoxxygenase with the second type of human 15S-lipoxxygenase (15-Lox-2). The top line shows the amino acid sequence (SEQ ID NO:4) deduced from the mouse 8S-lipoxxygenase cDNA, in alignment

with the amino acid sequence (SEQ ID NO:2) of the second type of human 15S-lipoxygenase. Five putative iron ligands are in boldface (H374, H379, H554, S558, I676 - 8-Lox; H374, H379, H554, S558, I676 - 15-Lox-2).

5        Figure 7 shows expression of 8S-lipoxygenase in vaccinia virus infected Hela cells. Cells sonicates were incubated with [1-<sup>14</sup>C]arachidonic acid (100 μM) for 30 min at room temperature, then extracted with methylene chloride and treated with triphenylphosphine in methanol  
10    to reduce HPETEs to HETEs.

Figure 7A: The products were analyzed by normal phase HPLC using an Alltech 5μ silica column (25 x 0.46 cm) and a solvent of hexane/isopropanol/glacial acetic acid (100:2:0.1, by volume) at a flow rate of 1.1 ml/min.  
15    The column effluent was monitored using a Hewlett-Packard 1040A diode array detector with an on-line Packard Flo-one radioactive detector.

Figure 7B: The chirality of the 8-HETE product was analyzed as the methyl ester derivative using a Chiralcel  
20    OD column (25 x 0.46 cm) and a solvent of hexane/isopropanol (100:2, by volume) at a flow rate of 1.1 ml/min.

Figure 7C shows Western analyses of 8S-lipoxygenase expressed in Hela cells, HEK cells and the enzyme from  
25    mouse skin. Lane 1: 15-Lox-1 expressed in HEK cells

(antibody does not recognize this protein). Lane 2: 15-  
Lox-2, HEK cells. Lane 3: 8S-lipoxygenase in HEK cells.  
Lane 4: PMA-treated mouse skin. Lane 5: 8S-lipoxygenase in  
Hela cells. Lane 6: 15-Lox-2 in Hela cells. All lanes were  
5 loaded with 5  $\mu$ g protein, except lane 4 had only 2.5  $\mu$ g  
protein.

Figure 8 shows linoleic acid metabolism by 8S-  
lipoxygenase expressed in vaccinia virus-infected HeLa  
cells. Metabolism studies with [ $^{14}$ C]linoleic acid (100  $\mu$ M)  
10 used the same incubation and analysis conditions as  
described in the legend to Figure 7.

Figure 8A: Normal phase-HPLC of the products,  
analyzed after reduction with triphenylphosphine.

Figure 8B: Chiral HPLC analysis of the 9-HODE methyl  
15 ester.

Figure 9 shows the effect of phorbol ester on 8S-  
lipoxygenase expression in mouse skin.

Figure 9A: Normal-phase HPLC analysis of 8S-  
lipoxygenase activity in homogenates of back skin of 7 -  
20 8-day-old black Swiss pups following 24 hr treatment with  
vehicle (acetone) or phorbol ester (50 nmol).

Figure 9B: Northern analysis.

Figure 9C: Western analysis.

Figure 10 shows multiple tissue Northern analysis of  
25 mouse 8S-lipoxygenase. A mouse tissue blot of mRNA

(Clontech) was probed with a 618 bp *EcoRV-BamHI* fragment of 8S-lipoxygenase cDNA.

#### Detailed Description of the Invention

5       The lipoxygenase metabolism of arachidonic acid occurs in specific blood cell types and epithelial tissues, and is activated in inflammation and tissue injury. In the course of studying lipoxygenase expression in human skin, a previously unrecognized enzyme was detected and characterized that at least partly accounts  
10       for the 15S-lipoxygenase metabolism of arachidonic acid in certain epithelial tissues. The cDNA was cloned from human hair roots, and expression of the mRNA was detected also in prostate, lung, and cornea; an additional sixteen human tissues, including peripheral blood leukocytes, were  
15       negative for the mRNA. The cDNA encodes a protein of 676 amino acids with a calculated molecular weight of about 76 kD. The amino acid sequence has approximately 40% identity to the known human 5S-, 12S- and 15S-lipoxygenases.

20       When expressed in human embryonic kidney (HEK) 293 cells, the new enzyme converts arachidonic acid exclusively to 15S-hydroperoxyeicosatetraenoic acid, while linoleic acid is less well metabolized. These features

contrast with the previously reported 15S-lipoxygenase which oxygenates arachidonic acid mainly at C-15, but also partly at C-12, and for which linoleic acid is an excellent substrate. The different catalytic activities and tissue distribution suggest a distinct function for the new enzyme compared to the previously reported human 15S-lipoxygenase.

It is known that human hair roots metabolize arachidonic acid (Henneicke-von Zepelin et al. (1991) *J. Invest. Dermatol.* 97:291-297), and that in addition to a relatively prominent synthesis of 12S-HETE and 15S-HETE, formation of minor amounts of 12R-HETE is detectable. (Baer et al. (1993) *J. Lipid Research* 34:1505-1514). Therefore freshly plucked human hair follicles were as a source of RNA for the RT-PCR experiments described in Example 1. As described in Example 1, these experiments led to the detection of a new lipoxygenase, a 15S-lipoxygenase (referred to herein as "15-Lox-2") with a distinctive distribution in tissues.

Definitions and Techniques Affecting

Gene Products and Genes

The present invention concerns DNA segments, isolatable from vertebrate tissue, and preferably mammalian tissue, which are free from genomic DNA and which are capable of conferring arachidonic acid metabolism activity in a recombinant host cell when incorporated into the recombinant host cell. As used herein, the term "mammalian tissue" refers to, among others, normal mammalian epithelial tissues, as exemplified by, but not limited to, human embryonic kidney (HEK) 293 cell lines. DNA segments capable of conferring arachidonic acid metabolism activity may encode complete lipoxxygenase gene products, cleavage products and biologically actively functional domains thereof.

The terms "lipoxxygenase gene product", "lipoxxygenase", "Lox", "15-Lox-2 gene product", "15-Lox-2", "8-Lox gene product" and "8-Lox" as used in the specification and in the claims refer to proteins having amino acid sequences which are substantially identical to the respective native lipoxxygenase amino acid sequences and which are biologically active in that they are capable of reacting with arachidonic acid or are capable of cross-reacting with an anti-Lox antibody raised against a lipoxxygenase, such as 15-Lox-2 or 8-Lox. Such sequences

are disclosed herein. The terms "lipoxygenase gene product", "lipoxygenase", "Lox", "15-Lox-2 gene product", "15-Lox-2", "8-Lox gene product" and "8-Lox" also include analogs of lipoxygenase molecules which exhibit at least some biological activity in common with native lipoxygenase, 15-Lox-2, or 8-Lox. Furthermore, those skilled in the art of mutagenesis will appreciate that other analogs, as yet undisclosed or undiscovered, may be used to construct lipoxygenase analogs. There is no need for a "lipoxygenase" or "Lox", or a "15-Lox-2" or "8-Lox" to comprise all, or substantially all, of the amino acid sequence of the native lipoxygenase genes. Shorter or longer sequences are anticipated to be of use in the invention.

The terms "lipoxygenase gene", "15-lox-2 gene" and "8-Lox gene" refer to any DNA sequence that is substantially identical to a DNA sequence encoding a lipoxygenase, 15-Lox-2 or 8-Lox as defined above. The terms also refer to RNA, or antisense sequences, compatible with such DNA sequences. A "lipoxygenase gene", a "15-lox-2 gene" or a "8-Lox gene" may also comprise any combination of associated control sequences.

The term "substantially identical", when used to define either a lipoxygenase, a 15-lox-2 or a 8-Lox amino acid sequence, or a lipoxygenase, a 15-lox-2 or a 8-Lox

nucleic acid sequence, means that a particular sequence, for example, a mutant sequence, varies from the sequence of a natural lipxygenase, 15-lox-2 or 8-Lox by one or more deletions, substitutions, or additions, the net effect of which is to retain at least some of biological activity of the lipxygenase, the 15-lox-2 or the 8-Lox protein. Alternatively, DNA analog sequences are "substantially identical" to specific DNA sequences disclosed herein if: (a) the DNA analog sequence is derived from coding regions of the natural lipxygenase, 15-lox-2 or 8-Lox gene; or (b) the DNA analog sequence is capable of hybridization of DNA sequences of (a) under moderately stringent conditions and which encode biologically active lipxygenase, 15-lox-2 or 8-Lox gene; or (c) the DNA sequences are degenerative as a result of the genetic code to the DNA analog sequences defined in (a) and/or (b). Substantially identical analog proteins will be greater than about 60% identical to the corresponding sequence of the native protein. Sequences having lesser degrees of similarity but comparable biological activity are considered to be equivalents. In determining nucleic acid sequences, all subject nucleic acid sequences capable of encoding substantially similar amino acid sequences are considered to be substantially

similar to a reference nucleic acid sequence, regardless of differences in codon sequences.

#### Percent Similarity

Percent similarity may be determined, for example, by  
5 comparing sequence information using the GAP computer  
program, available from the University of Wisconsin  
Geneticist Computer Group. The GAP program utilizes the  
alignment method of Needleman et al. 1970, as revised by  
Smith et al. 1981. Briefly, the GAP program defines  
10 similarity as the number of aligned symbols (i.e.  
nucleotides or amino acids) which are similar, divided by  
the total number of symbols in the shorter of the two  
sequences. The preferred default parameters for the GAP  
program include: (1) a unitary comparison matrix  
15 (containing a value of 1 for identities and 0 for non-  
identities) of nucleotides and the weighted comparison  
matrix of Gribskov et al., 1986, as described by Schwartz  
et al., 1979; (2) a penalty of 3.0 for each gap and an  
additional 0.01 penalty for each symbol and each gap; and  
20 (3) no penalty for end gaps.

The term "homology" describes a mathematically based  
comparison of sequence similarities which is used to  
identify genes or proteins with similar functions or  
motifs. Accordingly, the term "homology" is synonymous

with the term "similarity" and "percent similarity" as defined above. Thus, the phrases "substantial homology" or "substantial similarity" have similar meanings.

#### Nucleic Acid Sequences

5           In certain embodiments, the invention concerns the use of lipoxxygenase genes and gene products, such as the 15-lox-2 and 8-Lox gene products, that include within their respective sequences a sequence which is essentially that of a lipoxxygenase, 15-lox-2 or 8-Lox gene, or the  
10           corresponding proteins. The term "a sequence essentially as that of lipoxxygenase, 15-lox-2 or 8-Lox gene or gene product", means that the sequence substantially corresponds to a portion of a lipoxxygenase, 15-lox-2 or 8-Lox gene or gene product and has relatively few bases or  
15           amino acids (whether DNA or protein) which are not identical to those of a lipoxxygenase, 15-lox-2 or 8-Lox gene or gene product, (or a biologically functional equivalent of, when referring to proteins). The term  
20           "biologically functional equivalent" is well understood in the art and is further defined in detail herein. Accordingly, sequences which have between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids which are identical or functionally

equivalent to the amino acids of a lipoxxygenase, 15-lox-2 or 8-Lox gene or gene product, will be sequences which are "essentially the same".

5 Lipoxxygenase, 15-lox-2 and 8-Lox genes which have functionally equivalent codons are also covered by the invention. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, and also to refer to codons that encode biologically  
10 equivalent amino acids (see Table 1).

TABLE 1

Functionally Equivalent Codons.

Amino Acids				Codons			
5	Alanine	Ala	A	GCA	GCC	GCG	GCU
	Cysteine	Cys	C	UGC	UGU		
	Aspartic Acid	Asp	D	GAC	GAU		
	Glumatic acid	Glu	E	GAA	GAG		
	Phenylalanine	Phe	F	UUC	UUU		
10	Glycine	Gly	G	GGA	GGC	GGG	GGU
	Histidine	His	H	CAC	CAU		
	Isoleucine	Ile	I	AUA	AUC	AUU	
	Lysine	Lys	K	AAA	AAG		
	Leucine	Leu	L	UUA	UUG	CUA	CUC
15	Methionine	Met	M	AUG			
	Asparagine	Asn	N	AAC	AAU		
	Proline	Pro	P	CCA	CCC	CCG	CCU
	Glutamine	Gln	Q	CAA	CAG		
	Arginine	Arg	R	AGA	AGG	CGA	CGC
20	Serine	Ser	S	ACG	AGU	UCA	UCC
	Threonine	Thr	T	ACA	ACC	ACG	ACU
	Valine	Val	V	GUA	GUC	GUG	GUU
	Tryptophan	Trp	W	UGG			
	Tyrosine	Tyr	Y	UAC	UAU		
25							

It will also be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences which may, for example, include various non-coding

sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, i.e., introns, which are known to occur within genes.

The present invention also encompasses the use of  
5 DNA segments which are complementary, or essentially complementary, to the sequences set forth in the specification. Nucleic acid sequences which are "complementary" are those which are base-pairing according to the standard Watson-Crick complementarity rules. As  
10 used herein, the term "complementary sequences" means nucleic acid sequences which are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid segment in question  
15 under relatively stringent conditions such as those described herein.

Nucleic acid hybridization will be affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of  
20 the complementary strands, and the number of nucleotide base mismatches between the hybridizing nucleic acids, as will be readily appreciated by those skilled in the art. Stringent temperature conditions will generally include temperatures in excess of 30°C, typically in excess of  
25 37°C, and preferably in excess of 45°C. Stringent salt

conditions will ordinarily be less than 1,000 mM, typically less than 500 mM, and preferably less than 200 mM. However, the combination of parameters is much more important than the measure of any single parameter. (See, 5 e.g., Wetmur & Davidson, 1968).

Probe sequences may also hybridize specifically to duplex DNA under certain conditions to form triplex or other higher order DNA complexes. The preparation of such probes and suitable hybridization conditions are well 10 known in the art.

As used herein, the term "DNA segment" refers to a DNA molecule which has been isolated free of total genomic DNA of a particular species. Furthermore, a DNA segment encoding a lipoxxygenase, 15-lox-2 or 8-Lox gene product 15 refers to a DNA segment which contains lipoxxygenase, 15-lox-2 or 8-Lox coding sequences, yet is isolated away from, or purified free from, total genomic DNA of Homo sapiens. Included within the term "DNA segment" are DNA segments and smaller fragments of such segments, and also 20 recombinant vectors, including, for example, plasmids, cosmids, phages, viruses, and the like.

Similarly, a DNA segment comprising an isolated or purified lipoxxygenase, 15-lox-2 or 8-Lox gene refers to a DNA segment including lipoxxygenase, 15-lox-2 or 8-Lox 25 coding sequences isolated substantially away from other

naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein, polypeptide or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences and cDNA sequences. "Isolated substantially away from other coding sequences" means that the gene of interest, in this case, the lipxygenase, 15-lox-2 or 8-Lox gene, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

In particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences which encode a 15-Lox-2 protein that includes within its amino acid sequence the amino acid sequence of SEQ ID NO:2. In other particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences which encode a protein that includes within its amino acid

sequence the amino acid sequence of the 15-Lox-2 protein corresponding to human epithelial tissue.

In particular embodiments, the invention concerns isolated DNA segments and recombinant vectors  
5 incorporating DNA sequences which encode a 8-Lox protein that includes within its amino acid sequence the amino acid sequence of SEQ ID NO:4. In other particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences which  
10 encode a protein that includes within its amino acid sequence the amino acid sequence of the 8-Lox protein corresponding to mouse epithelial tissue.

It will also be understood that this invention is not limited to the particular nucleic acid and amino acid  
15 sequences of SEQ ID NOS:1, 2, 3 and 4. Recombinant vectors and isolated DNA segments may therefore variously include the 15-Lox-2 and 8-Lox encoding regions themselves, include coding regions bearing selected alterations or modifications in the basic coding region,  
20 or include encoded larger polypeptides which nevertheless include 15-Lox-2 or 8-Lox encoding regions or may encode biologically functional equivalent proteins or peptides which have variant amino acid sequences.

In certain embodiments, the invention concerns  
25 isolated DNA segments and recombinant vectors which encode

a protein or peptide that includes within its amino acid sequence an amino acid sequence essentially as set forth in SEQ ID NO:2 or SEQ ID NO:4. Naturally, where the DNA segment or vector encodes a full length 15-Lox-2 or 8-Lox gene product, the most preferred sequences are those which are essentially as set forth in SEQ ID NO:1 and SEQ ID NO:3 and which encode a protein that exhibits arachidonic acid reactivity in HEK 293 cells, as may be determined by HPLC analysis, as disclosed herein.

10       The term "a sequence essentially as set forth in SEQ ID NO:2" means that the sequence substantially corresponds to a portion of SEQ ID NO:2 and has relatively few amino acids which are not identical to, or a biologically functional equivalent of, the amino acids of SEQ ID NO:2.

15       The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein. Accordingly, sequences, which have between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids which are identical or functionally equivalent to the amino acids of SEQ ID NO:2, will be sequences which are "essentially as set forth in SEQ ID NO:2". The term "a sequence essentially set forth in SEQ ID NO:4" has a similar meaning.

20

In particular embodiments, the invention concerns gene therapy methods that use isolated DNA segments and recombinant vectors incorporating DNA sequences which encode a protein that includes within its amino acid sequence an amino acid sequence in accordance with SEQ ID NO:2 or in accordance with SEQ ID NO:4, SEQ ID NO:2 and SEQ ID NO:4 derived from epithelial tissue from Homo sapiens. In other particular embodiments, the invention concerns isolated DNA sequences and recombinant DNA vectors incorporating DNA sequences which encode a protein that includes within its amino acid sequence the amino acid sequence of the 15-Lox-2 protein from human epithelial tissue, or which encode a protein that includes within its amino acid sequence the amino acid sequence of the 8-Lox protein from mouse epithelial tissue.

In certain other embodiments, the invention concerns isolated DNA segments and recombinant vectors that include within their sequence a nucleic acid sequence essentially as set forth in SEQ ID NO:1, or a nucleic acid sequence essentially as set forth in SEQ ID NO:3. The term "essentially as set forth in SEQ ID NO:1" is used in the same sense as described above and means that the nucleic acid sequence substantially corresponds to a portion of SEQ ID NO:1, respectively, and has relatively few codons which are not identical, or functionally equivalent, to

the codons of SEQ ID NO:1, respectively. Again, DNA segments which encode gene products exhibiting arachidonic acid metabolism activity of the 15-Lox-2 and 8-Lox gene products will be most preferred. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, and also to refer to codons that encode biologically equivalent amino acids (see Table 1). The term "essentially as set forth in SEQ ID NO:3" has a similar meaning.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, enhancers, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, nucleic acid fragments may be prepared which include a short stretch complementary to SEQ ID NO:1 or SEQ ID NO:3, such as about 10 nucleotides, and which are up to 10,000 or 5,000 base pairs in length, with segments of 3,000 being

preferred in certain cases. DNA segments with total lengths of about 1,000, 500, 200, 100 and about 50 base pairs in length are also contemplated to be useful.

5 The DNA segments of the present invention encompass biologically functional equivalent 15-Lox-2 and 8-Lox proteins and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency which are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively,  
10 functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man  
15 may be introduced through the application of site-directed mutagenesis techniques, e.g., to introduce improvements to the antigenicity of the protein or to test 15-Lox-2 and 8-Lox mutants in order to examine arachidonic acid reactivity at the molecular level.

20 If desired, one may also prepare fusion proteins and peptides, e.g., where the 15-Lox-2 or 8-Lox coding regions are aligned within the same expression unit with other proteins or peptides having desired functions, such as for purification or immunodetection purposes (e.g., proteins

which may be purified by affinity chromatography and enzyme label coding regions, respectively).

Recombinant vectors form important further aspects of the present invention. Particularly useful vectors are contemplated to be those vectors in which the coding portion of the DNA segment is positioned under the control of a promoter. The promoter may be in the form of the promoter which is naturally associated with the 15-Lox-2 or 8-Lox gene(s), e.g., in epithelial cells, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR technology, in connection with the compositions disclosed herein.

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with a 15-Lox-2 or 8-Lox gene in its natural environment. Such promoters may include promoters isolated from bacterial, viral, eukaryotic, or mammalian cells. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type chosen for expression. The use of promoter and cell type

combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook et al., 1989, specifically incorporated herein by reference. The promoters employed  
5 may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides. Appropriate promoter systems contemplated for  
10 use in high-level expression include, but are not limited to, the vaccina virus promoter, which is more fully described below.

As mentioned above, in connection with expression embodiments to prepare recombinant 15-Lox-2 and 8-Lox  
15 proteins and peptides, it is contemplated that longer DNA segments will most often be used, with DNA segments encoding the entire 15-Lox-2 or 8-Lox protein, functional domains or cleavage products thereof, being most preferred. However, it will be appreciated that the use  
20 of shorter DNA segments to direct the expression of 15-Lox-2 and 8-Lox peptides or epitopic core regions, such as may be used to generate anti-15-Lox-2 or anti-8-Lox antibodies, also falls within the scope of the invention.

DNA segments which encode peptide antigens from  
25 about 15 to about 50 amino acids in length, or more

preferably, from about 15 to about 30 amino acids in length are contemplated to be particularly useful. DNA segments encoding peptides will generally have a minimum coding length in the order of about 45 to about 150, or to about 90 nucleotides. DNA segments encoding full length proteins may have a minimum coding length on the order of about 5,600 nucleotides for a protein in accordance with SEQ ID NO:2 or a minimum coding length on the order of about 10,300 nucleotides for a protein in accordance with SEQ ID NO:4.

Naturally, the present invention also encompasses DNA segments which are complementary, or essentially complementary, to the sequence set forth in SEQ ID NO:1 or the sequence set forth in SEQ ID NO:3. The terms "complementary" and "essentially complementary" are defined above. Excepting intronic or flanking regions, and allowing for the degeneracy of the genetic code, sequences which have between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of nucleotides which are identical or functionally equivalent (i.e. encoding the same amino acid) of nucleotides of SEQ ID NO:1 or to the nucleotides of SEQ ID NO:3, will be respectively sequences which are "essentially as set forth in SEQ ID NO:1" and will be sequences which are

"essentially as set forth in SEQ ID NO:3". Sequences which are essentially the same as those set forth in SEQ ID NO:1 or as those set forth in SEQ ID NO:3 may also be functionally defined as sequences which are capable of hybridizing to a nucleic acid segment containing the complement of SEQ ID NO:1 or to a nucleic acid segment containing the complement of SEQ ID NO:3 under relatively stringent conditions. Suitable relatively stringent hybridization conditions are described herein and will be well known to those of skill in the art.

#### Biologically Functional Equivalents

As mentioned above, modification and changes may be made in the structure of the lipxygenase proteins and peptides, including 15-Lox-2 and 8-Lox, described herein and still obtain a molecule having like or otherwise desirable characteristics. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, C-15 carbon or C-8 carbon of arachidonic acid. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence (or, of course, its underlying DNA

coding sequence) and nevertheless obtain a protein with like or even countervailing properties (e.g., antagonistic v. agonistic). It is thus contemplated by the inventors that various changes may be made in the sequence of the lipoxxygenase proteins and peptides, including 15-Lox-2 and 8-Lox, (or underlying DNA) without appreciable loss of their biological utility or activity.

It is also well understood by the skilled artisan that, inherent in the definition of a biologically functional equivalent protein or peptide, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. Biologically functional equivalent peptides are thus defined herein as those peptides in which certain, not most or all, of the amino acids may be substituted. Of course, a plurality of distinct proteins/peptides with different substitutions may easily be made and used in accordance with the invention.

It is also well understood that where certain residues are shown to be particularly important to the biological or structural properties of a protein or peptide, e.g., residues in active sites, such residues may not generally be exchanged. This is the case in the

present invention, where it any changes, for example, in  
an iron ligand moiety of 15-Lox-2 that render the peptide  
incapable of metabolism of arachidonic acid to 15S-  
Hydro(pero)xyeicosatetraenoic acid would result in a loss  
5 of utility of the resulting peptide for the present  
invention.

Amino acid substitutions, such as those which might  
be employed in modifying the lipxygenase proteins and  
peptides, including 15-Lox-2 and 8-Lox, described herein,  
10 are generally based on the relative similarity of the  
amino acid side-chain substituents, for example, their  
hydrophobicity, hydrophilicity, charge, size, and the  
like. An analysis of the size, shape and type of the  
amino acid side-chain substituents reveals that arginine,  
15 lysine and histidine are all positively charged residues;  
that alanine, glycine and serine are all a similar size;  
and that phenylalanine, tryptophan and tyrosine all have  
a generally similar shape. Therefore, based upon these  
considerations, arginine, lysine and histidine; alanine,  
20 glycine and serine; and phenylalanine, tryptophan and  
tyrosine; are defined herein as biologically functional  
equivalents.

In making such changes, the hydropathic index of  
amino acids may be considered. Each amino acid has been  
25 assigned a hydropathic index on the basis of their

hydrophobicity and charge characteristics, these are:  
isoleucine (+ 4.5); valine (+ 4.2); leucine (+ 3.8);  
phenylalanine (+ 2.8); cysteine/cystine (+ 2.5);  
methionine (+ 1.9); alanine (+ 1.8); glycine (-0.4);  
5 threonine (-0.7); serine (-0.8); tryptophan (-0.9);  
tyrosine (-1.3); proline (-1.6); histidine (-3.2);  
glutamate (-3.5); glutamine (-3.5); aspartate (-3.5);  
asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index  
10 in conferring interactive biological function on a protein  
is generally understood in the art (Kyte & Doolittle,  
1982, incorporated herein by reference). It is known that  
certain amino acids may be substituted for other amino  
acids having a similar hydropathic index or score and  
15 still retain a similar biological activity. In making  
changes based upon the hydropathic index, the substitution  
of amino acids whose hydropathic indices are within  $\pm 2$  is  
preferred, those which are within  $\pm 1$  are particularly  
preferred, and those within  $\pm 0.5$  are even more  
20 particularly preferred.

It is also understood in the art that the  
substitution of like amino acids can be made effectively  
on the basis of hydrophilicity. U.S. Pat. No. 4,554,101,  
incorporated herein by reference, states that the greatest  
25 local average hydrophilicity of a protein, as governed by

the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e. with a biological property of the protein. It is understood that an amino acid can be substituted for another having a  
5 similar hydrophilicity value and still obtain a biologically equivalent protein.

As detailed in U.S. Pat. No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+ 3.0); lysine (+ 3.0);  
10 aspartate (+ 3.0 $\pm$ 1); glutamate (+ 3.0 $\pm$ 1); serine (+ 0.3); asparagine (+ 0.2); glutamine (+ 0.2); glycine (0); threonine (-0.4); proline (-0.5 $\pm$ 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine  
15 (-2.3); phenylalanine (-2.5); tryptophan (-3.4) .

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within  $\pm 2$  is preferred, those which are within  $\pm 1$  are particularly preferred, and those  
20 within  $\pm 0.5$  are even more particularly preferred.

While discussion has focused on functionally equivalent polypeptides arising from amino acid changes, it will be appreciated that these changes may be effected by alteration of the encoding DNA; taking into

consideration also that the genetic code is degenerate and that two or more codons may code for the same amino acid.

#### Sequence Modification Techniques

Modifications to the lipxygenase proteins and  
5 peptides, including 15-Lox-2 and 8-Lox, described herein  
may be carried out using techniques such as site directed  
mutagenesis. Site-specific mutagenesis is a technique  
useful in the preparation of individual peptides, or  
biologically functional equivalent proteins or peptides,  
10 through specific mutagenesis of the underlying DNA. The  
technique further provides a ready ability to prepare and  
test sequence variants, for example, incorporating one or  
more of the foregoing considerations, by introducing one  
or more nucleotide sequence changes into the DNA. Site-  
15 specific mutagenesis allows the production of mutants  
through the use of specific oligonucleotide sequences  
which encode the DNA sequence of the desired mutation, as  
well as a sufficient number of adjacent nucleotides, to  
provide a primer sequence of sufficient size and sequence  
20 complexity to form a stable duplex on both sides of the  
deletion junction being traversed. Typically, a primer of  
about 17 to 25 nucleotides in length is preferred, with  
about 5 to 10 residues on both sides of the junction of  
the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art as exemplified by publications (e.g., Adelman et al., 1983). As will be appreciated, the technique typically employs a phage  
5 vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage (Messing et al., 1981). These phage are readily commercially available and their use is generally well known to those  
10 skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance  
15 herewith is performed by first obtaining a single-stranded vector or melting apart the two strands of a double stranded vector which includes within its sequence a DNA sequence which encodes, for example, the 15-Lox-2 and the 8-Lox gene. An oligonucleotide primer bearing the desired  
20 mutated sequence is prepared, generally synthetically, for example by the method of Crea et al. (1978). This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the  
25 synthesis of the mutation-bearing strand. Thus, a

heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected gene using site-directed mutagenesis is provided as a means of producing potentially useful 15-Lox-2, 8-Lox or other arachidonic acid metabolizing species and is not meant to be limiting as there are other ways in which sequence variants of these peptides may be obtained. For example, recombinant vectors encoding the desired genes may be treated with mutagenic agents to obtain sequence variants (see, e.g., a method described by Eichenlaub, 1979) for the mutagenesis of plasmid DNA using hydroxylamine.

#### Other Structural Equivalents

In addition to the lipxygenase peptidyl compounds described herein, the inventors also contemplate that other sterically similar compounds may be formulated to mimic the key portions of the peptide structure. Such compounds may be used in the same manner as the peptides of the invention and hence are also functional

equivalents. The generation of a structural functional equivalent may be achieved by the techniques of modeling and chemical design known to those of skill in the art. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

#### Introduction of Gene Products

Where the gene itself is employed to introduce the gene products, a convenient method of introduction will be through the use of a recombinant vector which incorporates the desired gene, together with its associated control sequences. The preparation of recombinant vectors is well known to those of skill in the art and described in many references, such as, for example, Sambrook et al. (1989), specifically incorporated herein by reference.

In vectors, it is understood that the DNA coding sequences to be expressed, in this case those encoding the lipoxxygenase gene products, are positioned adjacent to and under the control of a promoter. It is understood in the art that to bring a coding sequence under the control of such a promoter, one generally positions the 5' end of the transcription initiation site of the transcriptional reading frame of the gene product to be expressed between about 1 and about 50 nucleotides "downstream" of (i.e., 3'

of) the chosen promoter. One may also desire to incorporate into the transcriptional unit of the vector an appropriate polyadenylation site (e.g., 5'-AATAAA-3'), if one was not contained within the original inserted DNA.

5 Typically, these poly A addition sites are placed about 30 to 2000 nucleotides "downstream" of the coding sequence at a position prior to transcription termination.

While use of the control sequences of the specific gene (i.e., the 15-Lox-2 promoter for 15-Lox-2) will be  
10 preferred, there is no reason why other control sequences could not be employed, so long as they are compatible with the genotype of the cell being treated. Thus, one may mention other useful promoters by way of example, including, e.g., an SV40 early promoter, a long terminal  
15 repeat promoter from retrovirus, an actin promoter, a heat shock promoter, a metallothionein promoter, and the like.

As is known in the art, a promoter is a region of a DNA molecule typically within about 100 nucleotide pairs in front of (upstream of) the point at which transcription  
20 begins (i.e., a transcription start site). That region typically contains several types of DNA sequence elements that are located in similar relative positions in different genes. As used herein, the term "promoter" includes what is referred to in the art as an upstream  
25 promoter region, a promoter region or a promoter of a

generalized eukaryotic RNA Polymerase II transcription unit.

Another type of discrete transcription regulatory sequence element is an enhancer. An enhancer provides  
5 specificity of time, location and expression level for a particular encoding region (e.g., gene). A major function of an enhancer is to increase the level of transcription of a coding sequence in a cell that contains one or more transcription factors that bind to that enhancer. Unlike  
10 a promoter, an enhancer can function when located at variable distances from transcription start sites so long as a promoter is present.

As used herein, the phrase "enhancer-promoter" means a composite unit that contains both enhancer and promoter  
15 elements. An enhancer-promoter is operatively linked to a coding sequence that encodes at least one gene product. As used herein, the phrase "operatively linked" means that an enhancer-promoter is connected to a coding sequence in such a way that the transcription of that coding sequence  
20 is controlled and regulated by that enhancer-promoter. Means for operatively linking an enhancer-promoter to a coding sequence are well known in the art. As is also well known in the art, the precise orientation and location relative to a coding sequence whose transcription  
25 is controlled, is dependent inter alia upon the specific

nature of the enhancer-promoter. Thus, a TATA box minimal promoter is typically located from about 25 to about 30 base pairs upstream of a transcription initiation site and an upstream promoter element is typically located from about 100 to about 200 base pairs upstream of a transcription initiation site. In contrast, an enhancer can be located downstream from the initiation site and can be at a considerable distance from that site.

An enhancer-promoter used in a vector construct of the present invention can be any enhancer-promoter that drives expression in a cell to be transfected. By employing an enhancer-promoter with well-known properties, the level and pattern of gene product expression can be optimized.

For introduction of, for example, the 15-Lox-2 or 8-Lox genes, it is proposed that one will desire to preferably employ a vector construct that will deliver the desired gene to the affected cells. This will, of course, generally require that the construct be delivered to the targeted cells, for example, epithelial cells. It is proposed that this may be achieved most preferably by introduction of the desired gene through the use of a viral vector to carry either the 15-Lox-2 sequence or the 8-Lox sequence to efficiently infect the cells. These vectors will preferably be an adenoviral, a retroviral, a

vaccinia viral vector or adeno-associated virus. These vectors are preferred because they have been successfully used to deliver desired sequences to cells and tend to have a high infection efficiency.

5           Commonly used viral promoters for expression vectors are derived from polyoma, cytomegalovirus, Adenovirus 2, and Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also  
10 contains the SV40 viral origin of replication. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the viral origin of replication. Further, it is also  
15 possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

          The origin of replication may be provided either by  
20 construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the  
25 latter is often sufficient.

Where the 15-Lox-2 or 8-Lox genes themselves are employed it will be most convenient to simply use the wild type 15-Lox-2 gene or 8-Lox gene directly. However, it is contemplated that certain regions of either the 15-Lox-2 gene or the 8-Lox gene may be employed exclusively without employing the entire wild type 15-Lox-2 or 8-Lox gene. It is proposed that it will ultimately be preferable to employ the smallest region needed to regulate the metabolism of arachidonic acid to 15S-hydro(pero)xyeicosatetraenoic acid or to 8S-hydro(pero)xyeicosatetraenoic acid so that one is not introducing unnecessary DNA into cells which receive either a 15-Lox-2 gene construct or an 8-Lox gene construct. Techniques well known to those of skill in the art, such as the use of restriction enzymes, will allow for the generation of small regions of the 15-Lox-2 or 8-Lox genes. The ability of these regions to regulate the metabolism of arachidonic acid to 15S-hydro(pero)xyeicosatetraenoic acid or to 8S-hydro(pero)xyeicosatetraenoic acid can easily be determined by the assays reported in the Examples. In general, techniques for assessing metabolism of arachidonic acid to 15S-Hydro(pero)xyeicosatetraenoic acid or to 8S-hydro(pero)xyeicosatetraenoic acid are well known in the art.

Generation of Antibodies

In still another embodiment, the present invention provides an antibody immunoreactive with a polypeptide of the present invention. Preferably, an antibody of the invention is a monoclonal antibody. Means for preparing and characterizing antibodies are well known in the art (See, e.g., *Antibodies A Laboratory Manual*, E. Howell and D. Lane, Cold Spring Harbor Laboratory, 1988).

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide or polynucleotide of the present invention, and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster or a guinea pig. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given polypeptide or polynucleotide may vary in its immunogenicity. It is often necessary therefore to couple the immunogen (e.g., a polypeptide or polynucleotide) of the present invention with a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum

albumin or rabbit serum albumin can also be used as carriers.

Means for conjugating a polypeptide or a polynucleotide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, immunogenicity to a particular immunogen can be enhanced by the use of non-specific stimulators of the immune response known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen used of the production of polyclonal antibodies varies inter alia, upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies is monitored by sampling blood of the immunized animal at various points following immunization. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored.

In another aspect, the present invention contemplates a process of producing an antibody immunoreactive with a lipxygenase polypeptide, such as 15-Lox-2 or 8-Lox, the process comprising the steps of (a) 5 transfecting recombinant host cells with a polynucleotide that encodes that polypeptide; (b) culturing the host cells under conditions sufficient for expression of the polypeptide; (c) recovering the polypeptide; and (d) preparing antibodies to the polypeptide. Preferably, the 10 lipxygenase polypeptide is capable of metabolizing arachidonic acid. Even more preferably, the present invention provides antibodies prepared according to the process described above.

A monoclonal antibody of the present invention can 15 be readily prepared through use of well-known techniques such as those exemplified in U.S. Pat. No 4,196,265, herein incorporated by reference. Typically, a technique involves first immunizing a suitable animal with a selected antigen (e.g., a polypeptide or polynucleotide of 20 the present invention) in a manner sufficient to provide an immune response. Rodents such as mice and rats are preferred animals. Spleen cells from the immunized animal are then fused with cells of an immortal myeloma cell. Where the immunized animal is a mouse, a preferred myeloma 25 cell is a murine NS-1 myeloma cell.

The fused spleen/myeloma cells are cultured in a selective medium to select fused spleen/myeloma cells from the parental cells. Fused cells are separated from the mixture of non-fused parental cells, for example, by the addition of agents that block the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides. Where azaserine is used, the media is supplemented with hypoxanthine.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants for reactivity with an antigen-polypeptides. The selected clones can then be propagated indefinitely to provide the monoclonal antibody.

By way of specific example, to produce an antibody of the present invention, mice are injected intraperitoneally with between about 1-200 mg of an

antigen comprising a polypeptide of the present invention.

B lymphocyte cells are stimulated to grow by injecting the antigen in association with an adjuvant such as complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*).

At some time (e.g., at least two weeks) after the first injection, mice are boosted by injection with a second dose of the antigen mixed with incomplete Freund's adjuvant.

A few weeks after the second injection, mice are tail bled and the sera titered by immunoprecipitation against radiolabeled antigen. Preferably, the process of boosting and titering is repeated until a suitable titer is achieved. The spleen of the mouse with the highest titer is removed and the spleen lymphocytes are obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately  $5 \times 10^7$  to  $2 \times 10^8$  lymphocytes.

Mutant lymphocyte cells known as myeloma cells are obtained from laboratory animals in which such cells have been induced to grow by a variety of well-known methods. Myeloma cells lack the salvage pathway of nucleotide biosynthesis. Because myeloma cells are tumor cells, they can be propagated indefinitely in tissue culture, and are thus denominated immortal. Numerous cultured cell lines

of myeloma cells from mice and rats, such as murine NS-1 myeloma cells, have been established.

Myeloma cells are combined under conditions appropriate to foster fusion with the normal  
5 antibody-producing cells from the spleen of the mouse or rat injected with the antigen/polypeptide of the present invention. Fusion conditions include, for example, the presence of polyethylene glycol. The resulting fused cells are hybridoma cells. Like myeloma cells, hybridoma  
10 cells grow indefinitely in culture.

Hybridoma cells are separated from unfused myeloma cells by culturing in a selection medium such as HAT media (hypoxanthine, aminopterin, thymidine). Unfused myeloma cells lack the enzymes necessary to synthesize nucleotides  
15 from the salvage pathway because they are killed in the presence of aminopterin, methotrexate, or azaserine. Unfused lymphocytes also do not continue to grow in tissue culture. Thus, only cells that have successfully fused (hybridoma cells) can grow in the selection media.

20 Each of the surviving hybridoma cells produces a single antibody. These cells are then screened for the production of the specific antibody immunoreactive with an antigen/polypeptide of the present invention. Single cell hybridomas are isolated by limiting dilutions of the  
25 hybridomas. The hybridomas are serially diluted many

times and, after the dilutions are allowed to grow, the supernatant is tested for the presence of the monoclonal antibody. The clones producing that antibody are then cultured in large amounts to produce an antibody of the present invention in convenient quantity.

By use of a monoclonal antibody of the present invention, specific polypeptides and polynucleotide of the invention can be recognized as antigens, and thus identified. Once identified, those polypeptides and polynucleotide can be isolated and purified by techniques such as antibody-affinity chromatography. In antibody-affinity chromatography, a monoclonal antibody is bound to a solid substrate and exposed to a solution containing the desired antigen. The antigen is removed from the solution through an immunospecific reaction with the bound antibody. The polypeptide or polynucleotide is then easily removed from the substrate and purified.

#### Detecting a Polynucleotide or a Polypeptide of the Present Invention

Alternatively, the present invention provides a process of detecting a polypeptide of the present invention, wherein the process comprises immunoreacting the polypeptides with antibodies prepared according to the

process described above to form antibody-polypeptide conjugates, and detecting the conjugates.

5 In yet another embodiment, the present invention contemplates a process of detecting messenger RNA transcripts that encode a polypeptide of the present invention, wherein the process comprises hybridizing the messenger RNA transcripts with polynucleotide sequences that encode the polypeptide to form duplexes; and detecting the duplex. Alternatively, the present invention provides a process of detecting DNA molecules  
10 that encode a polypeptide of the present invention, wherein the process comprises hybridizing DNA molecules with a polynucleotide that encodes that polypeptide to form duplexes; and detecting the duplexes.

15

#### Screening Assays

In yet another aspect, the present invention contemplates a process of screening substances for their ability to affect arachidonic acid metabolism comprising the steps of providing a cell that contains a functional polypeptide of the present invention and testing the  
20 ability of selected substances to affect arachidonic acid metabolism in that cell.

Utilizing the methods and compositions of the present invention, screening assays for the testing of

candidate substances can be derived. A candidate substance is a substance which potentially can promote or inhibit arachidonic acid metabolism, by binding or other intramolecular interaction, with a lipxygenase polypeptide, such as 15-Lox-2 or 8-Lox, that metabolizes arachidonic acid.

A screening assay of the present invention generally involves determining the ability of a candidate substance to affect metabolism of arachidonic acid in a target cell, such as the screening of candidate substances to identify those that inhibit or promote metabolism of arachidonic acid. Target cells can be either naturally occurring cells known to contain a polypeptide of the present invention or transformed cell produced in accordance with a process of transformation set forth hereinbefore.

As is well known in the art, a screening assay provides a cell under conditions suitable for testing arachidonic acid metabolism. These conditions include but are not limited to pH, temperature, tonicity, the presence of relevant factors involved in arachidonic acid metabolism (e.g., metal ions such as for example  $\text{Ca}^{++}$ , growth factor, interleukins, or colony stimulating factors), and relevant modifications to the polypeptide such as glycosylation or prenylation. It is contemplated that a polypeptide of the present invention can be

expressed and utilized in a prokaryotic or eukaryotic cell. The host cell can also be fractionated into sub-cellular fractions where the receptor can be found. For example, cells expressing the polypeptide can be  
5 fractionated into the nuclei, the endoplasmic reticulum, vesicles, or the membrane surfaces of the cell.

pH is preferably from about a value of 6.0 to a value of about 8.0, more preferably from about a value of about 6.8 to a value of about 7.8 and, most preferably  
10 about 7.4. In a preferred embodiment, temperature is from about 20°C. to about 50°C., more preferably from about 30°C. to about 40°C. and, even more preferably about 37°C. Osmolality is preferably from about 5 milliosmols per liter (mosm/L) to about 400 mosm/l and, more preferably  
15 from about 200 milliosmols per liter to about 400 mosm/l and, even more preferably from about 290 mosm/L to about 310 mosm/L. The presence of factors can be required for the proper testing of arachidonic acid metabolism in specific cells. Such factors include, for example, the  
20 presence and absence (withdrawal) of growth factor, interleukins, or colony stimulating factors.

In one embodiment, a screening assay is designed to be capable of discriminating candidate substances having selective ability to interact with one or more of the  
25 polypeptides of the present invention but which

polypeptides are without a substantially overlapping activity with another of those polypeptides identified herein.

Screening Assays for a Polypeptide of the

5

Present Invention

The present invention provides a process of screening a biological sample for the presence of a lipoxxygenase polypeptide, such as 15-Lox-2 or 8-Lox. Preferably, the lipoxxygenase polypeptide reacts with  
10 arachidonic acid. A biological sample to be screened can be a biological fluid such as extracellular or intracellular fluid or a cell or tissue extract or homogenate. A biological sample can also be an isolated cell (e.g., in culture) or a collection of cells such as  
15 in a tissue sample or histology sample. A tissue sample can be suspended in a liquid medium or fixed onto a solid support such as a microscope slide.

In accordance with a screening assay process, a biological sample is exposed to an antibody immunoreactive  
20 with the polypeptide whose presence is being assayed. Typically, exposure is accomplished by forming an admixture in a liquid medium that contains both the antibody and the candidate polypeptide. Either the antibody or the sample with the polypeptide can be affixed  
25 to a solid support (e.g., a column or a microtiter plate).

The biological sample is exposed to the antibody under biological reaction conditions and for a period of time sufficient for antibody-polypeptide conjugate formation. Biological reaction conditions include ionic composition and concentration, temperature, pH and the like.

Ionic composition and concentration can range from that of distilled water to a 2 molal solution of NaCl. Preferably, osmolality is from about 100 mosmols/l to about 400 mosmols/l and, more preferably from about 200 mosmols/l to about 300 mosmols/l. Temperature preferably is from about 4°C. to about 100°C., more preferably from about 15°C. to about 50°C. and, even more preferably from about 25°C to about 40°C. pH is preferably from about a value of 4.0 to a value of about 9.0, more preferably from about a value of 6.5 to a value of about 8.5 and, even more preferably from about a value of 7.0 to a value of about 7.5. The only limit on biological reaction conditions is that the conditions selected allow for antibody-polypeptide conjugate formation and that the conditions do not adversely affect either the antibody or the polypeptide.

Exposure time will vary inter alia with the biological conditions used, the concentration of antibody and polypeptide and the nature of the sample (e.g., fluid

or tissue sample). Means for determining exposure time are well known to one of ordinary skill in the art. Typically, where the sample is fluid and the concentration of polypeptide in that sample is about  $10^{-10}\text{M}$ , exposure time is from about 10 minutes to about 200 minutes.

The presence of polypeptide in the sample is detected by detecting the formation and presence of antibody-polypeptide conjugates. Means for detecting such antibody-antigen (e.g., receptor polypeptide) conjugates or complexes are well known in the art and include such procedures as centrifugation, affinity chromatography and the like, binding of a secondary antibody to the antibody-candidate receptor complex.

In one embodiment, detection is accomplished by detecting an indicator affixed to the antibody. Exemplary and well known such indicators include radioactive labels (e.g.,  $^{32}\text{P}$ ,  $^{125}\text{I}$ ,  $^{14}\text{C}$ ), a second antibody or an enzyme such as horse radish peroxidase. Means for affixing indicators to antibodies are well known in the art. Commercial kits are available.

#### Screening Assay for Anti-Polypeptide Antibody

In another aspect, the present invention provides a process of screening a biological sample for the presence of antibodies immunoreactive with a lipoyxygenase polypeptide, such as 15-Lox-2 or 8-Lox. Preferably the

lipxygenase polypeptide reacts with arachidonic acid. In accordance with such a process, a biological sample is exposed to a lipxygenase polypeptide, such as 15-Lox-2 or 8-Lox, under biological conditions and for a period of time sufficient for antibody-polypeptide conjugate formation and the formed conjugates are detected.

Screening Assay for Polynucleotide That Encodes a Lipxygenase Polypeptide, such as 15-Lox-2 or 8-Lox

A DNA molecule and, particularly a probe molecule, can be used for hybridizing as an oligonucleotide probe to a DNA source suspected of encoding a lipxygenase polypeptide, such as 15-Lox-2 or 8-Lox. Preferably the lipxygenase polypeptide reacts with arachidonic acid. The probing is usually accomplished by hybridizing the oligonucleotide to a DNA source suspected of possessing a lipxygenase gene. In some cases, the probes constitute only a single probe, and in others, the probes constitute a collection of probes based on a certain amino acid sequence or sequences of the polypeptide and account in their diversity for the redundancy inherent in the genetic code.

A suitable source of DNA for probing in this manner is capable of expressing a polypeptide of the present invention and can be a genomic library of a cell line of

interest. Alternatively, a source of DNA can include total DNA from the cell line of interest. Once the hybridization process of the invention has identified a candidate DNA segment, one confirms that a positive clone  
5 has been obtained by further hybridization, restriction enzyme mapping, sequencing and/or expression and testing.

Alternatively, such DNA molecules can be used in a number of techniques including their use as: (1) diagnostic tools to detect normal and abnormal DNA  
10 sequences in DNA derived from patient's cells; (2) means for detecting and isolating other members of the polypeptide family and related polypeptides from a DNA library potentially containing such sequences; (3) primers for hybridizing to related sequences for the purpose of  
15 amplifying those sequences; (4) primers for altering native lipxygenase DNA sequences; as well as other techniques which rely on the similarity of the DNA sequences to those of the DNA segments herein disclosed.

As set forth above, in certain aspects, DNA sequence  
20 information provided by the invention allows for the preparation of relatively short DNA (or RNA) sequences (e.g., probes) that specifically hybridize to encoding sequences of a selected lipxygenase gene. In these aspects, nucleic acid probes of an appropriate length are  
25 prepared based on a consideration of the encoding sequence

for a polypeptide of this invention. The ability of such nucleic acid probes to specifically hybridize to other encoding sequences lend them particular utility in a variety of embodiments. Most importantly, the probes can  
5 be used in a variety of assays for detecting the presence of complementary sequences in a given sample. However, uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic  
10 constructions.

To provide certain of the advantages in accordance with the invention, a preferred nucleic acid sequence employed for hybridization studies or assays includes probe sequences that are complementary to at least a 14 to  
15 40 or so long nucleotide stretch of a nucleic acid sequence of the present invention, such as that shown in SEQ ID NO:1. A size of at least 14 nucleotides in length helps to ensure that the fragment is of sufficient length to form a duplex molecule that is both stable and  
20 selective. Molecules having complementary sequences over stretches greater than 14 bases in length are generally preferred, though, to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally  
25 prefer to design nucleic acid molecules having

gene-complementary stretches of 14 to 20 nucleotides, or even longer where desired. Such fragments can be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Pat. No. 4,683,202, herein incorporated by reference, or by introducing selected sequences into recombinant vectors for recombinant production.

Accordingly, a nucleotide sequence of the present invention can be used for its ability to selectively form duplex molecules with complementary stretches of the gene. Depending on the application envisioned, one employs varying conditions of hybridization to achieve varying degrees of selectivity of the probe toward the target sequence. For applications requiring a high degree of selectivity, one typically employs relatively stringent conditions to form the hybrids. For example, one selects relatively low salt and/or high temperature conditions, such as provided by 0.02M-0.15M NaCl at temperatures of 50°C to 70°C. Such conditions are particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one

seeks to isolate polypeptide coding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions are typically needed to allow formation of the heteroduplex. Under such  
5 circumstances, one employs conditions such as 0.15M-0.9M salt, at temperatures ranging from 20°C. to 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally  
10 appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a  
15 method of choice depending on the desired results.

In certain embodiments, it is advantageous to employ a nucleic acid sequence of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of  
20 appropriate indicator means are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one likely employs an enzyme tag such as urease, alkaline phosphatase or  
25 peroxidase, instead of radioactive or other

environmentally undesirable reagents. In the case of enzyme tags, calorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein are useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the sample containing test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions depend inter alia on the particular circumstances based on the particular criteria required (depending, for example, on the G+ C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe, etc.). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label.

Assay Kits.

In another aspect, the present invention contemplates diagnostic assay kits for detecting the presence of a polypeptide of the present invention in biological samples, where the kits comprise a first container containing a first antibody capable of immunoreacting with the polypeptide, with the first antibody present in an amount sufficient to perform at least one assay. Preferably, the assay kits of the invention further comprise a second container containing a second antibody that immunoreacts with the first antibody. More preferably, the antibodies used in the assay kits of the present invention are monoclonal antibodies. Even more preferably, the first antibody is affixed to a solid support. More preferably still, the first and second antibodies comprise an indicator, and, preferably, the indicator is a radioactive label or an enzyme.

The present invention also contemplates a diagnostic kit for screening agents. Such a kit can contain a polypeptide of the present invention. The kit can contain reagents for detecting an interaction between an agent and a receptor of the present invention. The provided reagent can be radiolabelled. The kit can contain a known

radiolabelled agent capable of binding or interacting with a receptor of the present invention.

In an alternative aspect, the present invention provides diagnostic assay kits for detecting the presence, in biological samples, of a polynucleotide that encodes a polypeptide of the present invention, the kits comprising a first container that contains a second polynucleotide identical or complementary to a segment of at least 10 contiguous nucleotide bases of, as a preferred example, SEQ ID NO:1 or SEQ ID NO:3.

In another embodiment, the present invention contemplates diagnostic assay kits for detecting the presence, in a biological sample, of antibodies immunoreactive with a polypeptide of the present invention, the kits comprising a first container containing a lipoxxygenase polypeptide, such as 15-Lox-2 or 8-Lox, that immunoreacts with the antibodies, with the polypeptide present in an amount sufficient to perform at least one assay. Preferably, the lipoxxygenase polypeptide metabolizes arachidonic acid. The reagents of the kit can be provided as a liquid solution, attached to a solid support or as a dried powder. Preferably, when the reagent is provided in a liquid solution, the liquid solution is an aqueous solution. Preferably, when the reagent provided is attached to a solid support, the solid

support can be chromatograph media or a microscope slide. When the reagent provided is a dry powder, the powder can be reconstituted by the addition of a suitable solvent. The solvent can be provided.

5           The following examples have been included to illustrate preferred modes of the invention. Certain aspects of the following examples are described in terms of techniques and procedures found or contemplated by the present inventors to work well in the practice of the  
10           invention. These examples are exemplified through the use of standard laboratory practices of the inventors. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the following examples are intended to be exemplary only and  
15           that numerous changes, modifications and alterations can be employed without departing from the spirit and scope of the invention.

#### EXAMPLE 1

##### ISOLATION OF A SECOND 15S-LIPOXYGENASE (15-LOX-2)

20           Preparation of total RNA, and cDNA synthesis - For each RNA preparation, about 50 human scalp hairs were plucked individually from a volunteer. About 30 hair

roots, mainly from anagen follicles (Baden et al. (1979) *J. Amer. Acad. Dermatol.* 1:121-122), were cut off and dropped into 1 ml of guanidinium thiocyanate solution, the lysis buffer from the RNeasy RNA extraction kit (Qiagen).

5 After a brief sonication using an ultrasonic probe (2 sec, twice), total RNA was extracted according to the manufacturer's instructions. Approximately 5-10  $\mu$ g of total RNA was recovered in 50  $\mu$ l of water. In some experiments, RNA was prepared from psoriatic scales using  
10 essentially the same procedure. Thirty microliter aliquots of RNA were used in 50 $\mu$ l reactions for first strand cDNA synthesis using either an oligo-dT-adaptor primer, random hexamer primers, or the Marathon RACE procedure (Clontech) as described previously (Brash et al. (1996) *J. Biol.Chem.*  
15 271, 20549-20557). One microliter aliquots of cDNA were used directly in PCR reactions.

PCR experiments - The primers encoded conserved sequences in animal and plant lipoxygenases. Two upstream primers encoded the sequence WLLAK (SEQ ID NO:5) from the  
20 middle of the lipoxygenase primary structure. This sequence forms the beginning of a long helix that crosses the center of the protein and includes two of the histidine iron ligands. The two upstream primers differed only in using alternative codons for the 3' lysine, AAA or  
25 AAG, and were designated as WLLAK-(AAA) and WLLAK-(AAG):

5'-GAC-GTC-TGG-YTi-YTi-GCi-AAA, (SEQ ID NO:6) or -AAG-3' (SEQ ID NO:7) (where i encodes inosine). The human 5S-lipoxygenase and the blood cell 15S-lipoxygenase are encoded as WLLAK-(AAA) (SEQ ID NO:6) (Matsumoto et al. (1988) *Proc. Natl. Acad. Sci. USA* 85, 26-30; Dixon et al. (1988) *Proc. Natl. Acad. Sci. USA* 85, 416-420; Sigal et al. (1988) *Biochem. Biophys. Res. Comm.* 157, 457-464), whereas the platelet 12-lipoxygenase uses WLLAK-(AAG) (SEQ ID NO:7) (Table 2) (Funk et al. (1990) *Proc. Natl. Acad. Sci. USA* 87, 5638-5642; Izumi et al. (1990) *Proc. Natl. Acad. Sci. USA* 87, 7477-7481). (One of the three papers on the human platelet 12-lipoxygenase reports a different sequence around this lysine, Yoshimoto et al. (1990) *Biochem. Biophys. Res. Comm.* 172, 1230-1235.)

For the first round PCR, each upstream primer was used in separate reactions against a set of downstream primers encoding an amino acid sequence that occurs seven amino acids downstream of the most 3' histidine ligand to the lipoxygenase iron on a second long helix. The sequence GQLDW (SEQ ID NO:8) occurs in the human 12S- and 15S-lipoxygenases beginning at amino acid position 546 and was encoded (with an additional three amino acids of consensus sequence on the 5' end) as 5'-CCA-AGT-GTA-CCA-RTC-NAG-YTG-NCC-3' (SEQ ID NO:9). The sequence GQYDW (SEQ ID NO:35) occurs in the equivalent position in the human

5S-lipoxygenase and this primer differed only in changing one amino acid code from leucine to tyrosine (5'-CCA-AGT-GTA-CCA-RTC-RTA-YTG-NCC-3') (SEQ ID NO:10).

The first round PCR reaction was primed with human hair follicle cDNA and in some experiments with cDNA prepared from psoriatic scales (1  $\mu$ l from a 50  $\mu$ l reaction using 5  $\mu$ g total RNA) per 50  $\mu$ l PCR reaction, and using 10 mM Tris, pH 8.3, 50 mM KCl, 3 mM MgCl<sub>2</sub> with 0.2 mM of each dNTP and 0.25  $\mu$ l (1.25 units) AmpliTaq DNA polymerase (Perkin Elmer) in a Perkin Elmer 480 thermocycler. After addition of cDNA at 80° (hot start), the PCR was programmed as follows: 94° for 2 min, 1 cycle; 50° for 1 min, 72° for 1 min, 94° for 1 min, 30 cycles; 72° for 10 min, 1 cycle, and then the block temperature was held at 4°C.

For second round PCR, the upstream primer was either retained as before (WLLAK-(AAA) (SEQ ID NO:6) or WLLAK-(AAG) (SEQ ID NO:7)), or changed to a nested upstream primer modified very slightly from that used by Funk and colleagues (Funk et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:5638-5642) for cloning of the human 12S-lipoxygenase and encoding the sequence XVDWLLAKXWVR (SEQ ID NO:36): 5'-TA-GTC-GAC-TGG-CTT-YTG-GCC-AAA-iiC-TGG-GTS-CG-3' (where S ("strong") encodes C or G) (SEQ ID NO:11). The downstream primer for all second round reactions (nested PCR) encoded the sequence ELQXWVR (SEQ ID NO:26) and included a *Bam*HI

restriction site at the 5' end: 5'-G-CGG-ATC-CCT-CCA-CCA-  
GGN-YTG-SAG-YTC-3' (SEQ ID NO:12). The second round PCR  
reactions used 1 $\mu$ l of 10-times dilute first round PCR  
products as cDNA and otherwise the conditions differed  
5 only in using either 55° or 58° as annealing temperature.

3' RACE and 5' RACE - The 3' sequence was obtained  
using established upstream sequence for the new human  
lipxygenase (first round: 5'-GGT-ATC-TAC-TAC-CCA-AGT-GAT-  
GAG-3' (SEQ ID NO:13); second round: 5'-TAC-CCA-AGT-GAT-  
10 GAG-TCT-GTC-3' (SEQ ID NO:14)) against a downstream primer  
based on the adaptor-linked oligo-dT primer used for cDNA  
synthesis, as described previously (Brash et al. (1996) *J.*  
*Biol.Chem.* 271:20549-20557). The 5' RACE was accomplished  
using the Marathon cDNA Amplication Kit (Clontech) (Brash  
15 et al. (1996) *J. Biol.Chem.* 271:20549-20557) using 4  $\mu$ g of  
total RNA from beard hair follicles. The gene-specific  
downstream primers were 5'-GAA-GAC-CTC-AGG-CAG-CAG-ATG-TG-  
3' (SEQ ID NO:15) and 5'-TC-ATG-GAA-GGA-GAA-CTC-GGC-AT-3'  
(SEQ ID NO:16). A full length clone was obtained by PCR  
20 using primers purified by HPLC (Brash et al. (1996) *J.*  
*Biol.Chem.* 271:20549-20557) and using a proof-reading  
mixture of Taq/Pwo DNA polymerases (Expand High Fidelity,  
Boehringer-Mannheim) as described (Brash et al. (1996) *J.*  
*Biol.Chem.* 271:20549-20557). The upstream primer encoded  
25 the N-terminus with a BamHI site added at the 5' end to

facilitate subcloning: 5' AC-GGA-TCC-AGC-ATG-GCC-GAG-TTC-  
AGG-GTC-AG 3' (SEQ ID NO:17), and the downstream primer  
encoded the C-terminus of the protein with an added  
5' *EcoRI* site to facilitate subcloning: 5' CGG-AAT-TCA-  
5 TGT-CAT-CTG-GGC-CTG-TGT-TCC 3' (SEQ ID NO:18). After a  
hot start at 80°C, the reaction conditions were 94°, 2 min,  
1 cycle; 58° for 30 sec, 72° for 1 min 30 sec, 96° 15 sec,  
3 cycles; 68° for 2 min, 96° 15 sec, 30 cycles; 72° 10 min,  
1 cycle; hold at 4°C.

10 Northern analysis - Two nylon membranes containing  
mRNA from human tissues (Clontech, Palo Alto, CA) were  
probed using a <sup>32</sup>P-labeled 1059 bp fragment of the new  
human lipoxxygenase prepared from the plasmid by PCR (with  
primers 5'-TG-CCT-CTC-GCC-ATC-CAG-CT-3' (SEQ ID NO:19) and  
15 5' TG-TTC-CCC-TGG-GAT-TTA-GAT-GGA-3') (SEQ ID NO:20) and  
labeled by Rediprime random priming (Amersham). After  
hybridization in ExpressHyb solution (Clontech) at 68°C for  
1 hr, the membranes were washed finally in 0.1 x SSC/0.1%  
SDS at 50°C for 40 min and exposed to film.

20 Detection of the cDNA in human cornea - RNA was  
prepared using Tri Reagent (Molecular Research Center,  
Inc., Cincinnati, OH) from corneal epithelial cells  
scraped from eye bank corneas unsuitable for  
transplantation. The RNA samples were treated with DNase  
25 1, then reverse transcribed to cDNA. PCR reactions were

run with human cornea cDNA as template, and also with rabbit cornea cDNA and buffer alone as negative controls. Additional negative controls using RNA without the reverse transcriptase step confirmed the absence of DNA contaminants in the samples. Two pairs of primers were used: GGT-ATC-TAC-TAC-CCA-AGT-GAT-GAG (SEQ ID NO:21) with 5'-TGCGATGTCATCTGGGCCTGT-3' (SEQ ID NO:22) giving a 589 bp product (# 1), and from the 3' untranslated region (UTR), #2: 5'-AACTCACCCCCACCACCATACACA-3' (SEQ ID NO:23) with 5'-TTCCCGCCTCCATCTCCCAAAGT-3' (SEQ ID NO:24) giving a 351 bp product (# 2). Both reactions were run using an annealing temperature of 65° in the PCR. Northern analysis of eye tissues used approximately 1 µg of poly A-selected RNA and the same hybridization protocol as given above.

DNA sequencing - PCR products were subcloned into the pCR2.1 vector (Invitrogen) and sequenced using the Oncor Fidelity manual dideoxy chain termination method or by automated sequencing on a ABI Prism 310 Genetic analyzer and fluorescence-tagged dye terminator cycle sequencing (Perkin Elmer).

Expression of cDNA, HPLC analysis of lipoxygenase metabolism - The PCR products corresponding to the open reading frame of the cDNA were subcloned into the pCDNA3 vector (Invitrogen), or in some experiments ligated directly into pCR3 (Invitrogen), and expressed by

transient transfection in human embryonic kidney (HEK) 293 cells as described (Funk et al. (1996) *J. Biol. Chem.* 271, 23338-23344). Following incubation with substrate (100  $\mu$ M [1- $^{14}$ C]arachidonic acid or [1- $^{14}$ C]linoleic acid) for 30 min at 37°C, products were extracted using the Bligh and Dyer procedure (Bligh et al. (1959) *Can. J. Biochem. Physiol.* 37:911-917) and the extracts were analyzed by reversed-phase HPLC, straight phase HPLC and chiral column analysis (Brash et al. (1990) *Method. Enzymol.* 187:187-192).

10        Results of PCR experiments - As described more fully in Experimental Procedures and summarized in Table 2, a PCR strategy was developed using sets of degenerate upstream and downstream primers that would resolve the known 5S-, 12S-, and 15S-lipoxygenases into separate  
15        tubes. The reactions were run under non-stringent conditions to permit detection of related sequences. After two rounds of reactions (nested PCR, see Experimental Procedures), successful amplification was expected to give a PCR product of approximately 500 bp.

20        When the reactions were carried out using different human hair root cDNAs as template, bands of  $\approx$ 500 bp were evident in tubes corresponding to several of the original combinations of primer. Many of the bands were found to represent the known 12S- and 15S-lipoxygenase sequences.  
25        These two cDNAs were successfully resolved into separate

PCR reactions by making use of their different codon usages for lysine 344 at the 3' end of the upstream primer (Table 2, and Experimental Procedures). Over 60 clones from the first two primer combinations in Table 2 were categorized as 12- or 15-lipoxygenase by sequencing and/or restriction enzyme digest with *ApaI* and *HindIII*.

Particular attention was paid to the 500 bp product obtained from the fourth primer set in Table 2, as this combination of sequences is not found in the three previously cloned human lipoxygenases. Of 41 clones with the correct sized insert, 39 cut with *ApaI* as expected of the human 12S-lipoxygenase. These clones appeared to correspond to 12S-lipoxygenase cDNA that had annealed to the slightly mismatched primers under the non-stringent conditions of PCR; a limited number were sequenced and all were identical to the human 12S-lipoxygenase. Two of the 41 positive clones did not cut with *ApaI* or *HindIII*, and sequencing indicated these clones represented a new lipoxygenase cDNA. The complete cDNA sequence of this new lipoxygenase was extended by 3' RACE and 5' RACE, and full length clones corresponding to the open reading frame were obtained by PCR. Two of the active clones (see below) were fully sequenced. The percent identity to the reported amino acid sequences of the 5S-, 12S- and 15S-lipoxygenases are approximately 44% to the 5-lipoxygenase,

and 38-39% to the 12- and 15-lipoxygenases. Fig. 1 shows the deduced amino acid sequence (SEQ ID NO:1) in alignment with the 15S-lipoxygenase of human blood cells (SEQ ID NO:25) (Sigal et al. (1988) *Biochem. Biophys. Res. Comm.* 157, 457-464).

Results of Expression studies - Initially, five full length clones were expressed in HEK 293 cells and the lipoxygenase activity evaluated by incubation with [<sup>14</sup>C]arachidonic acid followed by HPLC analysis. Three of the PCR clones expressed with equivalent activity. The active clones made a single product, identified as 15-HETE (after reduction of the HPETE) on the basis of its retention time on reversed-phase HPLC (Fig. 2A) and SP-HPLC, and its characteristic uv spectrum (Ingram et al. (1988) *Lipids* 23:340-344); it was exclusively the 15S enantiomer as determined by chiral column analysis (Fig. 2B). The same product was formed following expression in Hela cells and Cos cells, and in these experiments another twenty clones, eight active, were evaluated. Addition of calcium (2 mM) or ATP (2 mM) to the incubation media had no significant effect on enzymatic activity.

Differences from the 15S-lipoxygenase of blood cells - Applicants looked carefully for any 12-HETE or other HETE by-products of the new 15S-lipoxygenase and unexpectedly, found none. This is in sharp contrast to

the 15S-lipoxygenase of human blood cells that was analyzed in the same experiments; as reported before, the blood cell 15S-lipoxygenase forms 10-20% 12S-HETE in addition to 15S-HETE (Bryant et al. (1982) *J. Biol. Chem.* 257:6050-6055).

A comparison of the metabolism of arachidonic acid and linoleic acid revealed a second significant difference between the two enzymes. Linoleic acid is an excellent substrate for the blood cell 15S-lipoxygenase (Soberman et al. (1985) *J. Biol. Chem.* 260:4508-4515); in applicants' experiments it was metabolized more extensively than arachidonic acid. Although the new 15-lipoxygenase did metabolize linoleic acid, it was not as good a substrate. In two experiments, linoleic acid was 11% and 37% metabolized by the new enzyme, while the respective values for arachidonic acid were 30% and 83% conversion.

Expression in other tissues - Multiple tissue Northern blots showed fourteen tissues negative for the new 15S-lipoxygenase mRNA (heart, brain, placenta, liver, skeletal muscle, kidney, pancreas, spleen, thymus, testis, ovary, small intestine, colon, and peripheral blood leukocytes) and two distinctly positive (Fig. 3). The positive tissues, lung and prostate, showed a transcript estimated as 2.5 - 3 kb, compatible with the established size of the cDNA (2.7 kb). Applicants also checked for

the presence in cornea (originally because of a suspected connection to 12R-HETE synthesis). As determined by RT-PCR, human cornea is positive for the new lipoxygenase mRNA (Fig. 4a), and Northern analysis confirmed the presence of the new lipoxygenase transcript (Fig. 4b).

The human lipoxygenases can be distinguished by their positional specificity, by other distinctive features of their catalytic activities such as their ability to metabolize C18 fatty acid substrates, by their cellular distribution, and functionally, in their physiological roles. Funk, C. D. (1993) *Prog. Nuc. Acid Res. Mol. Biol.* 45:67-98. The new 15S-lipoxygenase characterized herein has a distinctive substrate specificity, a unique tissue distribution, and a different physiological role from the previously known human 15S-lipoxygenase.

The primary structure of the new enzyme has the features typical of a lipoxygenase. It has about 40% amino acid sequence identity to the blood cell 15S-lipoxygenase and other reported mammalian lipoxygenases.

The sequence contains the absolutely conserved iron-binding histidines and the carboxy terminal isoleucine that also functions as an iron ligand (Boyington et al. (1993) *Science* 260:1482-1486; Minor et al. (1996) *Biochemistry* 35:10687-10701). One difference from other

members of the lipoxygenase gene family is a change in the putative fifth iron ligand, normally a histidine or asparagine (N693 in the soybean L1 enzyme (Boyington et al. (1993) *Science* 260:1482-1486; Minor et al. (1996) *Biochemistry* 35:10687-10701), H544 in the human blood cell 15S-lipoxygenase (Sigal et al. (1988) *Biochem. Biophys. Res. Comm.* 157:457-464). In the new human lipoxygenase the equivalent residue is changed to a serine (S558).

10 Catalytically the enzyme differs from the blood cell 15S-lipoxygenase in two important respects: it oxygenates more exclusively at the 15 carbon, and linoleic acid is a relatively poor substrate. These two features of the new 15S-lipoxygenase, the high positional specificity and the  
15 preference for arachidonic acid, have a parallel among 12-lipoxygenases in the properties of the 12S-lipoxygenase of platelets (Nugteren, D. H. (1975) *Biochim. Biophys. Acta* 380:299-307; Hada et al. (1991) *Biochim. Biophys. Acta* 1083:89-93). Given this analogy in catalytic activities,  
20 it is likely that the new enzyme will be a comparatively poor metabolizer of esterified fatty acids, in contrast to the blood cell 15S-lipoxygenase (Schewe et al. (1975) *FEBS Lett.* 60:149-153; Murray et al. (1988) *Arch. Biochem. Biophys.* 265:514-523).

The four tissues in which the new enzyme is located, skin, lung, prostate and cornea, are all reported sites of 15-HETE synthesis. In human skin, applicants have now established the occurrence of both types of 15S-lipoxygenase. In lung, the 15-HETE synthesis has been ascribed to the blood cell type of 15-lipoxygenase (Sigal et al. (1992) *Am. J. Physiol.* 262:L392-398) and this enzyme has been detected by immunohistochemistry (Nadel et al. (1991) *J. Clin. Invest.* 87:1139-1145; Shannon et al. (1991) *Am. J. Physiol.* 261:L399-405; Shannon et al. (1993) *Am. Rev. Respir. Dis.* 147:1024-1028). But, clearly the possibility that the new 15-lipoxygenase contributes to the synthesis in certain cell types should be re-examined. Applicants' finding of the mRNA in prostate is compatible with the reports by Oliw and colleagues of the occurrence of 15-lipoxygenase in prostasomes, components of semen secreted by the prostate gland. Oliw et al. (1989) *Biochim. Biophys. Acta* 1002:283-291; Oliw et al. (1993) *J. Reprod. Fertil.* 99, 195-199. Similarly, our detection of the cDNA in cornea is in accord with metabolism studies by Oliw and coworkers; they established that human cornea synthesizes 15S-HETE from [<sup>14</sup>C]arachidonic acid. Liminga et al. (1994) *Biochim. Biophys. Acta* 1210:288-296. Additional studies using immunohistochemistry indicated expression of the blood

cell type of 15S-lipoxygenase in human cornea. Liminga et al. (1994) *Exp. Eye Res.* 59, 313-321. In cornea, as in skin, it is likely that both types of 15S-lipoxygenase are expressed.

5           It appears that this enzyme is a lipoxygenase specific for certain epithelial tissues. Based on the Northern result on colon and small intestine, the enzyme is not expressed in all epithelia, but the tissues in which it is identified so far are epithelial or have a significant epithelial component. As described in Example ,  
10           2, the new human enzyme is related in primary structure to the phorbol ester inducible 8S-lipoxygenase of mouse skin. Thus, regulation of the expression of the new human enzyme is a significant feature of its involvement in the  
15           pathophysiology of skin and other tissues.

Table 2 - Primers (first round PCR) to resolve human lipoxygenases

Upstream Primer	Downstream Match to known Primer <sup>a</sup> lipoxygenase		Reference for known lipoxygenase
WLLAK- (AAA) (SEQ ID NO:6)	GQLDW (SEQ ID NO:8)	15S-lipoxygenase	Sigal et al. (1988)

	WLLAK- (AAG)	GQLDW 12S-lipoxygenase	Funk et al.
	(SEQ ID NO:7)	(SEQ ID NO:8)	(1990); Izumi
			et al. (1990)
5	WLLAK- (AAA)	GQYDW 5S-lipoxygenase	Matsumoto et
	(SEQ ID NO:6)	(SEQ ID NO:35)	al. (1988);
			Dixon et al.
			(1988)
	WLLAK- (AAG)	GQYDW NONE	NONE
	(SEQ ID NO:7)	(SEQ ID NO:35)	

10

<sup>a</sup> All second round PCR reactions used the nested primer ELQXWWR (SEQ ID NO:26 and SEQ ID NO:12) described in Experimental Procedures.

## EXAMPLE 2

### 15 MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF A PHORBOL ESTER-INDUCIBLE 8S-LIPOXYGENASE (8-LOX) FROM MOUSE SKIN

As described above, in the course of studies on HETE synthesis in skin, a second type of 15S-lipoxygenase from  
20 human skin was cloned. This enzyme is different from the well known reticulocyte-type of 15S-lipoxygenase in that it oxygenates arachidonic acid purely at C-15 and linoleic acid is a relatively poor substrate. Continuing with the

abbreviations adopted above, the reticulocyte-type of 15S-lipoxygenase is referred to as 15-Lox-1 and the enzyme that is an aspect of this instant invention is referred to as 15-Lox-2 in this example.

5        It was not clear *a priori* what is the animal homologue of the new human lipoxygenase, 15-Lox-2. In searching for a potential murine homologue a series of PCR reactions using mouse skin were performed. This led to the detection of a new mouse cDNA that is characterized in  
10       this example.

      One of the effects of topical application of phorbol ester to mouse skin is the induction of an 8S-lipoxygenase in association with the inflammatory response. This example describes the molecular cloning and  
15       characterization of this enzyme. The cDNA was isolated by PCR from mouse epidermis and subsequently from a mouse epidermal cDNA library. The cDNA encodes a protein of 677 amino acids with a calculated molecular weight of 76 kDa. The amino acid sequence has 78% identity to 15-Lox-2, and  
20       approximately 40% to other mammalian lipoxygenases. When expressed in vaccinia virus-infected Hela cells, the mouse enzyme converts arachidonic acid exclusively to 8S-hydroperoxyeicosatetraenoic acid, while linoleic acid is converted to 9S-hydroperoxy-linoleic acid in lower  
25       efficiency.

Phorbol ester treatment of mouse skin is associated with strong induction of 8S-lipoxygenase mRNA and protein. By Northern analysis, expression of 8S-lipoxygenase mRNA was also detected in brain. Immunohistochemical analysis of phorbol ester-treated mouse skin showed the strongest reaction to 8S-lipoxygenase in the differentiated epidermal layer, the stratum granulosum. The inducibility of this enzyme is likely a characteristic feature of the mouse 8S-lipoxygenase and its human 15S-lipoxygenase homologue.

Preparation of mouse epidermal total RNA, and cDNA synthesis - Phorbol ester (PMA, 10 nmol) dissolved in 50 ml of acetone was applied topically onto dorsal skin of 6 - 7-day-old mice. At 21 - 24 h after PMA-treatment, the mice were euthanized, and epidermis was prepared from the frozen dorsal skin as previously described (Hughes et al. (1991) *Biochim. Biophys. Acta* 1081:347-354). The frozen epidermis was dropped into guanidinium thiocyanate solution, the lysis buffer from the RNeasy RNA extraction kit (QIAGEN). After a brief sonication using an ultrasonic probe (2 sec, twice), total RNA was extracted according to the manufacturer's instructions. Approximately 50 mg of total RNA was recovered in 50 ml of water. Twenty microliter aliquots were used in 50 ml reactions for first strand cDNA synthesis using an oligo-

dT-adaptor primer (Brash et al. (1996) *J. Biol. Chem.* 271:20949-20957).. One microliter aliquots of cDNA were used directly in PCR reactions.

PCR cloning of epidermal lipoxygenase cDNA - initial

5 PCR clone - Two upstream degenerate primers encoded the sequence DVWLLAK (SEQ ID NO:27). The two primers differed only in using alternative codons for the 3' lysine, AAA or AAG, and they are referred to as WLLAK-(AAA) (SEQ ID NO:6) and WLLAK-(AAG) (SEQ ID NO:7). For the first round PCR  
10 reaction, each upstream primer was used in separate reactions against a set of downstream primers encoding three amino acid sequences beginning GQ that occur seven amino acids downstream of the most 3' histidine ligand to the iron: the sequence GQLDW (SEQ ID NO:8) occurs in  
15 mammalian 12S- and 15S-lipoxygenases, GQYDW (SEQ ID NO:35) occurs in 5S-lipoxygenases, and GQFDS (SEQ ID NO:28) occurs in the new human 15S-lipoxygenase, 15-Lox-2. The primer sequences are the same as those described in Example 1 above, except for the new degenerate downstream  
20 primer encoding GQFDS (SEQ ID NO:28): 5'-CCA-AGC-GCA-SSA-RTC-RAA-YTG-NCC (where S, "strong", encodes C or G) (SEQ ID NO:29). For the second round nested PCR reaction, the upstream primer was retained as before [WLLAK-(AAA) (SEQ ID NO:6) or WLLAK-(AAG) (SEQ ID NO:7)], while the  
25 downstream primer was changed in all reactions to encode

the sequence ELQXWWR (SEQ ID NO:26). After the second round PCR, only the reaction that originally used the WLLAK-(AAG) (SEQ ID NO:7) and GQFDS primers (SEQ ID NO:29) yielded a visible PCR product. This product was 500 bp in size. The first round PCR reaction was primed with cDNA from phorbol ester-treated mouse epidermis, 1 ml per 50 ml PCR reaction (from a 50 ml cDNA synthesis using 20 mg total RNA), and using 10 mM Tris, pH 8.3, 50 mM KCl, 3 mM MgCl<sub>2</sub>, with 0.2 mM of each dNTP and 0.25 ml (1.25 units) of AmpliTaq DNA polymerase (Perkin Elmer) in a Perkin Elmer 480 thermocycler. After the addition of the cDNA at 80 °C (hot start), the PCR was programmed as follows: 94 °C for 2 min, 1 cycle; 50 °C for 1 min, 72 °C for 1 min, 94 °C for 1 min, 30 cycles; 72 °C for 10 min, 1 cycle, and then the block temperature was held at 4 °C. The second round reaction was primed with the equivalent of 0.1 ml of the first round reaction products (added as a 10-fold dilution). The protocol was 94 °C for 2 min, 1 cycle; 58 °C for 1 min, 72 °C for 1 min, 94 °C for 1 min for 30 cycles; the protocol was completed with one cycle at 72 °C for 10 min, and then the block temperature was held at 4 °C.

3'-RACE and 5'-RACE - The 3' sequence was obtained using established upstream sequence 5' G-AGC-TTT-GTC-TCT-GAA-ATA-GTC-AG 3' (SEQ ID NO:30) against a downstream

primer based on the adaptor-linked oligo-dT primer used for cDNA synthesis (Brash et al. (1996) *J. Biol. Chem.* 271:20949-20957). The 5' RACE was accomplished using a kit from GIBCO BRL according to the manufacturer's instructions. The gene-specific downstream primers were 5' GTG-AGG-AAT-CAA-TAG-CTT-GAA-GAG 3' (SEQ ID NO:31), and 5' G-ATG-TGT-GAC-AGC-CTC-ATG-GAT-G 3' (SEQ ID NO:32).

Full-length clones obtained by PCR - The upstream primer encoded the N-terminus with a *HindIII* site added at the 5' end to facilitate subcloning: 5' C-AAG-CTT-AGG-AGG-ATG-GCG-AAA-TGC-AGG 3' (SEQ ID NO:33), and the downstream primer encoded the C-terminus of the protein with an added 5' *EcoRI* site: 5' G-GAA-TTC-ATG-TTA-GAT-GGA-GAC-ACT-GTT 3' (SEQ ID NO:34). These two primers were purified by HPLC with the DMT protecting groups on (Brash et al. (1996) *J. Biol. Chem.* 271:20949-20957). After deprotection they were used in PCR reactions with a proof-reading mixture of Taq/Pwo DNA polymerase (Expand High Fidelity, Boehringer-Mannheim) according to the manufacturer's instructions. The reaction conditions were 94 °C, 2 min, 1 cycle; 58 °C for 30 sec, 72 °C for 1 min 30 sec, 96 °C for 15 sec, 3 cycles; 68 °C for 2 min, 96 °C 15 sec, 30 cycles; 72 °C for 10 min, 1 cycle; hold at 4 °C.

DNA Sequencing - cDNAs were sequenced using the Oncor Fidelity manual dideoxy chain termination method or by

automated sequencing on a ABI Prism 310 Genetic analyzer and fluorescence-tagged dye terminator cycle sequencing (Perkin Elmer).

HPLC Analysis of Lipoxygenase Metabolism - The  
5 lipoxygenase metabolism of [1-<sup>14</sup>C]arachidonic acid or [1-<sup>14</sup>C]linoleic acid was evaluated essentially as described previously (Hughes et al. (1991) *Biochim. Biophys. Acta* 1081:347-354). Following incubation with 100 mM of substrate, products were extracted using the Bligh and  
10 Dyer procedure (Bligh et al. (1959) *Can. J. Biochem. Physiol.* 37:911-917), and the extracts were analyzed by RP-HPLC, SP-HPLC and chiral column analysis (Brash et al. (1990) *Methods Enzymol.* 187:187-192). The hydroperoxide products were reduced with triphenylphosphine, methylated  
15 with diazomethane, purified by SP-HPLC, and then the stereochemistry was analyzed using a Chiralcel OD column.

Expression of Mouse 8s-lipoxygenase Clones - The PCR products corresponding to the open reading frame of the cDNA were ligated directly into pCR3.1 (Invitrogen), and  
20 expressed by transient transfection in HeLa cells using VTF-7, a recombinant vaccinia virus containing the T7 RNA polymerase gene (Blakely et al. (1991) *Anal. Biochem.* 194:302-308), or in human embryonic kidney (HEK) 293 cells using the adenovirus VA RNA gene. Funk et al. (1996) *J.*  
25 *Biol. Chem.* 271:23338-23344. In the former system, cells

plated at  $1 \times 10^6$  cells/35 mm well 48 h earlier were transfected with 1 mg of plasmid DNA and 3 mg of lipofectin, and harvested after 12 hours. In the HEK system, cells plated at  $1 \times 10^6$  cells/10 cm dish 24 h earlier were transfected with 10 mg of plasmid DNA by the calcium phosphate method, and harvested after two to three (2-3) days. Funk et al. (1996) *J. Biol. Chem.* 271:23338-23344. The harvested cells were sonicated on ice, and the resulting homogenates were incubated with 100 mM [1- $^{14}$ C]arachidonic acid or [1- $^{14}$ C]linoleic acid for 45 min at room temperature. The metabolites were extracted and analyzed as described above.

Screening of cDNA Library - The library was a commercial 1 Unizap.XR skin cDNA library prepared using poly(A)+ RNA isolated from whole skin of C57/Black female mice (Stratagene). It was screened with a 347 bp BsaMI fragment of the mouse 8S-lipoxygenase cDNA (PCR clone) as probe.

Northern Analysis - Poly(A)+ RNA was prepared from PMA- or acetone-treated frozen dorsal skin using TRI REAGENT<sup>®</sup> (Molecular Research Center, Inc.) and Oligotex<sup>™</sup> (Qiagen) according to the manufacturers' instructions. The poly(A)+ RNA was electrophoresed in 1% agarose/formaldehyde gel and blotted to a Hybond-H<sup>+</sup> nylon membrane (Amersham). The membrane was hybridized with  $^{32}$ P-

labeled DNA probe (complementary with a 0.6-kb *EcoRV/BamHI* fragment of mouse epidermal 8*S*-lipoxygenase) prepared using the Multiprime DNA labeling kit and Rapid-hybridization buffer (Amersham), and then washed according to the manufacturer's specifications. Blots were exposed to Fuji x-ray film at -80°C. Mouse cyclophilin cDNA was used as a house-keeping gene to access loading of RNA.

Western Analysis - After quantitation by Bradford assay (Bio-Rad), protein was separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred electrophoretically to Hybond ECL nitrocellulose membranes (Amersham). These were probed using a rabbit polyclonal antibody raised against the human 15-Lox-2. This antibody recognises 15-Lox-2 and the mouse 8*S*-lipoxygenase, but not the human reticulocyte type of 15*S*-lipoxygenase, as more fully described below. Donkey anti-rabbit Ig linked with horseradish peroxidase (Amersham) was the secondary antibody. Specifically bound protein was detected by chemiluminescence using the ECL Western blotting detection reagents (Amersham).

Immunohistochemical Analysis - The dorsal and tail skin of six to seven (6-7)-day-old mice were treated with acetone or PMA (10 nmol for dorsal skin, 2 nmol for tail skin). After 24 h, the animals were euthanized and the dorsal and tail skin were washed with soap and then rinsed

thoroughly with water. Whole dorsal and tail skin was immersion-fixed for 24 h in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), dehydrated in ethanolic solutions and xylenes, and embedded in paraffin.

5 Skin sections were deparaffinized, rehydrated in graded alcohols. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide/methanol for 20 min followed by incubation in 10% goat serum for 20 min. Sections were incubated at room temperature for 1 h in a 1/2500 dilution  
10 of either primary rabbit antisera that was used for Western analyses of 8S-lipoxygenase or pre-immune sera. After rinsing in PBS, sections were incubated with the biotinylated secondary antisera and peroxidase-labeled tertiary antisera supplied with an ABC Elite kit (Vector  
15 Corp., Gyrtingame, CA) followed by visualization of immunoprecipitate with DAB chromagen (Biogenix, Ban Ramon, CA).

Results of molecular cloning by RT-PCR - A series of PCR reactions were carried out with cDNA prepared from  
20 phorbol ester-treated mouse skin as template, and using degenerate primers based on well conserved sequences in mammalian lipoxygenases. The primers were identical to those used in the cloning of a novel 15S-lipoxygenase (15-Lox-2) from human skin as described in Example 1, with the  
25 addition of an extra downstream primer that better

represented the sequence of the new human enzyme. After running the protocol of nested PCR reactions, a strong band of the expected size of 500 bp was obtained in one of the reactions that used the new downstream primer (see  
5 above for details). The sequence of this PCR product showed a striking homology to the human lipoxxygenase sequence. The remainder of the mouse cDNA was cloned by conventional 3'- and 5'-RACE. cDNA corresponding to the open reading frame was prepared by PCR using a proof-  
10 reading mixture of *Taq/Pwo* as DNA polymerase. Eight of these clones were selected for expression studies. Seven clones were fully sequenced.

Subsequently, a partial cDNA sequence was used to screen a mouse skin cDNA library and two full length cDNAs  
15 of 3.2 kb were isolated and sequenced. These were identical to each other and also matched exactly one of the PCR products in the open reading frame (Figure 5). Figure 6 shows an alignment with the human 15-Lox-2. The sequences are 78% identical at both the DNA and protein  
20 levels.

Transient expression in HEK and Hela cells - The library clone was obtained relatively late in this study, and therefore much of the expression work described here was carried out using several of the PCR products. It  
25 became apparent very early on that there is some problem.

in expression of this mouse lipoxxygenase. Using a standard transient expression system in HEK 293 cells, it was only very occasionally that applicants detected enzymatic activity in the expressed mouse lipoxxygenase.

5 Positive controls using the human reticulocyte-type of 15S-lipoxxygenase (15-Lox-1) or the second type of human 15S-lipoxxygenase (15-Lox-2) were run in every experiment, and these cDNAs always expressed with readily detectible activity. In the few instances when active mouse  
10 lipoxxygenase was obtained in HEK cell expression, the enzyme converted arachidonic acid to 8S-hydroperoxyeicosatetraenoic acid (8S-HPETE).

Consistent expression of the mouse skin lipoxxygenase was obtained using HeLa cells infected with vaccinia virus encoding the T7 RNA polymerase. Blakely et al. (1991)  
15 *Anal. Biochem.* 194:302-308. In this system, using sonicated cells from a 35 mm well, typically 30-40% of added arachidonic acid (100  $\mu$ M) was converted to 8S-HPETE as the sole enzymatic product (Figure 7). The percentage  
20 conversion of arachidonic acid in this system was always similar to that obtained using the human 15-Lox-2 as a positive control. Active enzyme was obtained using several of the PCR clones (that encode one, two, or three different amino acids from the library clone, Figure 5  
25 description) and the library clone itself.

Using the vaccinia expression system, linoleic acid was found to be a substrate for the mouse 8S-lipoxygenase although the conversion was two to three (2-3)-fold lower compared to arachidonic acid. The enzyme converted  
5 linoleic acid exclusively to 9S-HODE (Figure 8).

Effect of phorbol ester on expression of 8S-lipoxygenase in mouse skin - The expression level of 8S-lipoxygenase in mouse skin is known to be strongly strain-dependent. Fischer et al. (1987) *Cancer Res.* 47:3174-  
10 3179; Fischer et al. (1987) *Carcinogenesis* 8:421-424; Fischer et al. (1988) *Cancer Res.* 48:658-664. Also, the highest activity is inducible in six to ten (6-10)-day-old animals. Gschwendt et al. (1986) *Carcinogenesis* 7:449-  
15 455; Fürstenberger et al. (1991) *J. Biol. Chem.* 266:15738-15745. Applicants examined several strains of mice and observed major differences in the level of constitutive expression (with no phorbol ester) and in the level after phorbol ester treatment. For example, using the Sencar strain applicants observed high constitutive 8S-  
20 lipoxygenase activity in six to ten (6-10)-day-old pups, with little extra induction by phorbol ester. The results shown here were obtained using a mixed breed of black Swiss animals that have low constitutive activity of 8S-lipoxygenase and exhibit strong induction with phorbol  
25 ester. Using six to ten (6-10)-day-old pups, the inducing

effect of phorbol ester clearly is related to induction of both mRNA and protein (Figure 9).

Cellular localization of 8S-lipoxygenase in mouse skin - The localization of mouse 8S-lipoxygenase protein in dorsal skin and tail skin was observed and characterized by immunohistochemical analysis. A thickened hyperproliferative epidermis after PMA treatment was observed. An increase in 8S-lipoxygenase is due to an expansion in cellularity in the stratum granulosum compartment of the epidermis. Baseline staining of the stratum granulosum for 8S-lipoxygenase in skin receiving vehicle alone (acetone) was performed. The absence of immunoreactivity after incubation of PMA treated skin with pre-immune antisera was also observed.

Thus, expression of the 8S-lipoxygenase protein was examined in normal mouse skin following treatment with phorbol ester in acetone (PMA) or acetone alone using the strain of black Swiss animals responsive to PMA. The histological analysis of skin from two differing body locations (thin dorsal skin and thick tail skin) revealed a marked hyperproliferative response to PMA and a diminished response to the acetone vehicle alone. Most notable was an increase in the number of differentiated cells within the outer epidermal compartment, the stratum granulosum. The net result was more 8S-lipoxygenase

positive cells in the the PMA treated samples as compared to the samples receiving acetone alone. No immunoreactivity was detected in any samples reacted with pre-immune serum. Hair follicles positioned within the underlying dermis also showed positive staining for 8S-lipoxygenase in differentiated cell layers. Staining in these locations did not show a modulation in response to topical treatment with phorbol ester.

Tissue distribution of 8S-lipoxygenase - As the related human 15S-lipoxygenase, 15-Lox-2, is expressed in prostate, an activity assay was used (HPLC analysis of products formed from [1-<sup>14</sup>C]arachidonic acid) to examine for 8S-lipoxygenase activity in mouse prostate. Using young adult males of 8 weeks of age, high levels of cyclooxygenase and 12S-lipoxygenase activities were found in the prostate, but no 8S-lipoxygenase products were detected. Occurrence of the 8S-lipoxygenase transcript was examined in several different tissues by Northern analysis. This revealed expression of 8S-lipoxygenase transcript in mouse brain, with no detectible expression in heart, spleen, lung, liver, skeletal muscle, kidney and testis (Figure 10).

Mouse 8S-lipoxygenase cDNA was cloned by PCR using primers related to the characterized human 15S-lipoxygenase (15-Lox-2) also described herein. These two

lipxygenases have 78% amino acid identity, and the differences are mainly conservative substitutions. The two enzymes have 30 - 45% identity to other mammalian lipxygenases. The primary structure of the mouse 8S-lipxygenase contains the absolutely conserved iron-binding histidines of lipxygenases and the C-terminal isoleucine that is also an iron ligand. Boyington et al. (1993) *Science* 260:1482-1486; Minor et al. (1996) *Biochemistry* 35:10687-10701. A notable feature of the mouse 8S-lipxygenase primary structure is the presence of a serine at amino acid position 558 as the putative 5th iron ligand. Minor et al. (1996) *Biochemistry* 35:10687-10701. The equivalent residue in all other lipxygenases is either a histidine or asparagine, with the exception of the human 15-Lox-2 in which a serine is also present. Based on the sequence similarity, the 8S-lipxygenase is the mouse homologue of the human 15-Lox-2.

Initially, there was difficulty in studying the mouse enzyme as reliable expression of active lipxygenase could not be obtained using a conventional HEK cell system. Chen et al. (1995) *J. Biol. Chem.* 270:17993-17999; Chen et al. (1994) *J. Biol. Chem.* 269:13979-13987. The problem was solved by use of the recombinant vaccinia virus in a co-transfection system in Hela cells. In this procedure, the cells are co-transfected with the plasmid cDNA and

vaccinia virus encoding the T7 RNA polymerase. The virus protein induces high level expression via the T7 promoter upstream of the lipoxxygenase cDNA. The cells are harvested after 12 hours. In this system, the mouse enzyme expressed with equivalent activity to either the 15-Lox-1 or 15-Lox-2 positive controls. Each of these lipoxxygenases was expressed at a much higher level in the viral infected Hela cells than in the other procedure using the HEK cells.

The Western results show clearly that HEK cells produce the 8S-lipoxxygenase protein, although at lower levels than the positive controls (Figure 7C). Whether this is a transfection problem, or related to translation or protein stability is not resolved. A similar poor expression of activity of the mouse epidermal-type of 12S-lipoxxygenase was found in HEK cells. Funk et al. (1996) *J. Biol. Chem.* 271:23338-23344. The lipoxxygenase proteins expressed in HEK and HeLa cells were indistinguishable in size by Western analysis. The very same preparations of 8S-lipoxxygenase plasmid cDNAs that failed to express active 8S-lipoxxygenase in HEK cells were expressed with good activity in the vaccinia system. Changing the vector from pCR3 to pCDNA3 had no effect on HEK cell expression. Extracts of Hela cells (+/- viral infection or vector alone) did not restore catalytic activity to HEK cells

transfected with 8S-lipoxygenase. Furthermore, changing the vaccinia virus system over to HEK cells failed to induce expression of 8S-lipoxygenase, whereas a 15-Lox-2 positive control gave easily measureable 15S-lipoxygenase activity. HEK cells may lack some factor that helps the effective expression of certain lipoxygenase enzymes.

Linoleic acid was converted with about 3-fold lower efficiency than arachidonic acid by the 8S-lipoxygenase. It was, however, specifically oxygenated to 9S-HPODE and likely participates in the biosynthesis of this product in vivo. Linoleic acid is an abundant polyunsaturated fatty acid in mouse skin and thus, potentially, this substrate is available. Ziboh et al. (1988) *Prog. Lipid Res.* 27:81-105. Lehmann and colleagues noted that the levels of 8-HETE and 9-HODE in mouse skin tend to change in parallel. Both are strikingly elevated in mouse skin papillomas are lower than normal in skin carcinomas. Lehmann et al. (1992) *Anal. Biochem.* 204:158-170. The 9S chirality of the product from linoleic acid is one criterion that could be used to assess the contribution of the 8S-lipoxygenase to formation of 9-HODE in mouse skin. The main cyclooxygenase product from linoleic acid is the enantiomeric 9R-HODE (Hamberg et al. (1980) *Biochim. Biophys. Acta* 617:545-547), while non-enzymatic reactions would give racemic product.

Northern and Western analyses, as well as the activity assay, showed that PMA treatment strongly induced *de novo* synthesis of mouse 8S-lipoxygenase in the dorsal skin of the outbred mice used in this experiment. The histochemical analyses further defined the effect of PMA. Immunoreactive 8S-lipoxygenase protein was most prominent in a layer of differentiated epidermis, the stratum granulosum. The thickness of this cell layer increased markedly following 24 hours of treatment with PMA. An increase in the number of cells that produce 8S-lipoxygenase, therefore, is one of the causes of the increased 8S-lipoxygenase activity induced by PMA.

In the Northern analysis using a multiple tissue blot, 8S-lipoxygenase mRNA was detected clearly in brain, but not in the other seven tissues examined. Both the stratum granulosum of the epidermis and the neuronal tissues of the central nervous system were originally derived from the same ectodermal layer in early embryonic development, and both represent highly differentiated cell types. Occurrence of the 8S-lipoxygenase transcript in brain was unexpected as lipoxygenase-catalyzed formation of 8-HETE has not been reported in neuronal tissues. The negative reaction in liver is of interest in relation to the reported activity of 8S-HETE as a strong activator of the peroxisome proliferator-activated receptor, PPAR- $\alpha$ .

Yu et al. (1995) *J. Biol. Chem.* 270:23975-23983. In liver, there is the possibility of synthesis of 8-HETE via the microsomal cytochrome P-450 system, although *in vitro* this gives a nearly racemic 8-HETE product. Capdevila et al. (1986) *Biochem. Biophys. Res. Commun.* 141:1007-1011.

The absence of 8S-lipoxygenase signal in the Northern analysis of liver, could, however, be related to the lack of induction in normal tissue. The same issue applies to the absence of detectible 8S-lipoxygenase activity in normal mouse prostate from young adult males. Although the human homologue of the mouse 8S-lipoxygenase, 15-Lox-2, was readily detectible in human prostate, as described in Example 1, the pooled human sample would include tissue from older subjects, the majority of which are expected to exhibit benign prostatic hyperplasia. Oesterling, J. E. (1996) *Prostate* 6:67-73. The induction of 8S-lipoxygenase in mouse skin by phorbol ester certainly is thus a striking feature of this enzyme.

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(1) GENERAL INFORMATION:

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(D) SOFTWARE: WORD PERFECT 6.1 and ASCII

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(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

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(A) LENGTH: 2685

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(2) INFORMATION FOR SEQ ID NO:2:  
(i) SEQUENCE CHARACTERISTICS:  
40 (A) LENGTH: 676  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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WGAVERFVSEIIGIYPSDESQDDRELQAWVREIFSKGFLNQESSGIPSSLETREALVQYVTMVIFTCSAKHAASAGG  
EDSCAWPNLPSPMQLPPPTSKGLATCEGFIATLPPVNAATCDVILALWLLSKEPGDQRPLGTYPDEHFTEAPRRSTATF  
QSRLAQISRGIERNRGLVLPYTYLDPLIENSVS

(2) INFORMATION FOR SEQ ID NO:3:  
(i) SEQUENCE CHARACTERISTICS:

55

- (A) LENGTH: 3125
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

10 ATGGCGAAATGCAGGGTGAAGATATCCACGGGGGAAGCCTGTGGGGCTGGCACATGGGACAAAGTGTCTGTGAGCATCGT  
GGGAACCCACGGAGAGAGCCCTTAGTACCTCTGGACCATCTGGGCAAGGAGTTGAGCGCCGGTGTGAAGAAGACTTCG  
AGGTGACGCTTCCCAGGACGTAGGCACTGTGCTGATGCTGCGAGTCCACAAAGCACCCTCGGAAGTGTCCCTCCGCTT  
ATGCTTTCCGTTCTGATGCCTGGTTCTGCCGCTGGTTCGAGCTGGAGTGGTACCTGGGGCTGCATCCACTTCCCCTG  
15 TTATCAGTGGCTGGAAGGGGCGGGGAGCTGGTGTGAGAGAGGGAGCAGCAAAGGTGTCTGGCAAGACCATCACCTA  
CACTGCAGGATCAGCGCCAGAGGAGCTTGAGTCCAGGCAGAGATGTACAGCTGGAAGACTTACATTGAAGGTTGGCT  
CGCTGCCTTGACCAGGAGACTGTGAAAGACTTGGACCTCAACATCAAGTACTCTGCGATGAAGAATGCCAAACTCTTCTT  
TAAAGCCCACTCCGCTATACGGAGCTGAAAGTCAAAGGGCTCCTGGACCGCACAGGACTTGGAGGAGTCTGAGGGAGA  
20 TGAGAAGGCTGTTAACTTCCGCAAGACTCCAGCAGCAGAGTATGTGTTGCACACTGGCAGGAAGATGCCTTCTCGCC  
TCCAGTTCCCTAAATGGCATCAACCCGGTCTGATTGCGCGCTGTACAGTCTCCCAAACAACCTCCCGGTCACTGATGA  
AATGGTGGCCCCAGTGTGGGCCCTGGAACCACTGTGAGGCTGAGTTGGAGAAGGGCTCCCTGTTCTTGGTGGATCATG  
GGATTCTTTCTGGAGTCCACACCAACATCTCAATGGAAAGCCTCAGTTCTCTGACGCCCGATGACCTGTACACCA  
AGCTCAGGGTCCGGACCCCTGCTTCCATTGCCATCCAGCTCAACAGACTCCCGGCCAGACAACCCCATCTTCTGCC  
25 CAGCGATGACAGTGGGACTGGTGTCTGGCCAAGACCTGGGTTGCAATTCTGAGTTTACATCCATGAGGCTGTACAC  
ATCTGCTGCATGCCCATCTGATTCCAGAAGCTTTGCTTGGCCACATTAGGTGAGTGCCTAGGTGTACCCCTCTCTT  
AAGCTATTGATTCTCACATTCGGTACACACTGCACATCAACACGCTTGCCCGGGAGCTGCTCGTTGCCCTGGGAAGT  
GATAGACAAGTCCACAGGCCCTGGCACTGGGGGATTCTCTGACCTGATAAAGAGAAACATGGAGCAGTGAAGTACTCTG  
30 TCCTGTGTCTCCCTGAAGATATCCGAGCCGAGGTGTGGAAGACATCCAGGCTACTATTACCGAGATGATGGGATGCAG  
ATCTGGGGGGCAATAAAGAGCTTTGTCTCTGAAATAGTCAGCATCTACTATCCAAGTGACACATCCGTCGAAGATGACCA  
AGAGCTCCAGGCCTGGGTGAGGGAGATCTTCTGAGGGCTTCTCGGCCGAGAAAGCTCAGGTATGCCCTCCTTGTGG  
ATACCCGGGAAGCCCTGGTCCAGTATATCACCATGGTGATATTACCTGCTCAGCCAAGCATGCAGTGTGACGTTAGGC  
35 CAGTTCGACTCTTGTGTTGGATGCCAATCTGCCACCTACCATGCAGCTACCACCACCTACTTCCAAAGGCCAGGCCCG  
GCCTGAGAGTTTCATAGCCACGCTCCCAGCAGTTAATTGTCAGTTATCACATCATTGCTCTCTGGCTGCTAAGCGCAG  
AACCTGGGGACCAAGGCCCTGGGCCATATCCAGATGAACACTTCACAGAGGATGCCCGCCGCGGAGCGTGGCTGCC  
TTCAGAGAAAGCTGATCCAGATCTCAAGGGCATCAGGGAGAGGAACCGAGGCTGGCACTGCCCTACACCTACCTGGA  
40 TCCCTCCCTCATTGAGAACAGTGTCTCCATCTAACATCTTGGAGAAGACAGTCTGTGTGACATATAGAATCTTGACCA  
TGCTCTCCAGGCTAAGTCCCGTATGCTTCTCTGGACAACCAAGCCCATCTTACACACACACACACACACACACACC  
TAATAAATCGAAACAGAAAACCTAAATCCACAGAAAGCAAGATCTCACACAGCAGAGAGCCATCCAAATGTTTGG  
45 GACCTGAGCTTCAGCTCTGATTACGGCTTGTCTGGTTTGTCTTCTATTCCATTAAACATGGACGGTAAACAGAA  
AGCACAGAACCTGGTTCACTGCACAAAGCCATGAGATCTACCCCTCACCTGACACAAAGGCAGCTATCATACAGGCTT  
ATCAGGAACACAGGAATTTGTCCAATCAAAGCCTACCCATAGGTCCATCGTGACCTACGACCTCACACTGGCATGCTTT  
AGCTTTGAGAAGGGATTACTGGAGTCAGGTACGAAGAGAAGGACAGGACGAAGGCATGGCTCCATGTGGAAGAACATATC  
50 TGCTCTTCCAGATGACCAGGGTAGCTCAGGCCATGTGTCTTCACTTCACTCCAGAGGTCTCTAGTGGCCATGAAGACTCCA  
GGCATTCAAGGGATATACAGTAGACACCAAAATTATACTTTTAAAGAGAGAGGATGGGCTGGAGAGATGGCTCAGCGGT  
TAAGAGCACTGACTGCTCTTCCAGAGATCCTGAGTCAATTTCCAGCAACCATGCTGGCTCACAACCATCTGTATGG  
GATTCCGATGCCCTCTTCTGGCGTGTCTGAAGACAGCAGTGTATGCACATATATAAAATAAATAAATCTTTAAAAAAC  
AAAACAGAGAGAGAGGACATGCTACCATTTCTACCTCACTTCTTCAAAGCCACCCTAAAGTGAATTTGTGAACAGGT  
CCCCTTTGAGAGAGTTAGAAGATATTCTCAAACCTCTAATACCTTCACATCTAAATCCATCTTCATTCCAAATTTCCA  
45 ATATTTATATACACTCTCAGTTTGGTGGGTGAGGGGTTGTTTTTGTGTTGGTTGGTTGGTTGGGTTTTGTTTTG  
TTTTGATTTTGTCTCTGGTTCAGACTCCATGGACGTTTATTAATGTATAAATGAGTTTATCCAAAAA

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

50

- (A) LENGTH: 677
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

55

MAKCRVRVSTGEACGAGTWDKVSIVSIVGTHGESPLVPLDHLGKEFSAGAEEDFEVTLPODVGTVMMLRVHKAPPEVSLPL  
MSFRSDAWFCRWFELEWLPGAALHFPCYOWLEAGELVLRGAAGVSWQDHHPTLQDQROKELESQKMYSWKTYIEGW  
RCLDHETVKDLNLNIKYSAMKNAKLFKAHSAYTELKVGLLDRTGLWRSREMRRLLFNFRKTPAAEYVFAHWQEDAFFA  
SQFLNGINPVLIRRHSLPNHFPVTDEMVAAPVLPVLPGLSLQAELEKGSFLVDHGLSGVHTNINLNGKPFSAAPMTLLHQ

5 SSGSGPLLPIAIQLKQTPGPDNPIFLPSDDTWDLAKTWVRNSEFYIHEAVTHLLHAHLIPEVFALATLRQLPRCHPLF  
KLLIPHIRYTLHINTLARELLVAPGKLIDKSTGLTGGFSDLIKRNMEQLNYSVLCLPEDIRARGVEDIPGYYRDDGMQ  
IWGAIKSFVSEIVSIYPSDTSVQDDQELQAWVREIFSEGFLGRESSGMPSLDTREALVQYITMVIFTCSAKHAHVSSG  
QFDSCVWMPNLPPTMQLPPPTSKGQARPESFIATLPAVNSSSYHIALWLLSAEPGDQRPLGHYPDEHFTEDAPRRSVAA  
FQRKLIQISKGIRERNRGLALPYTYLDPPLIENSVSI

- (2) INFORMATION FOR SEQ ID NO:5:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 5
- (B) TYPE: amino acid
- 10 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Trp Leu Leu Ala Lys  
1 5

- 15 (2) INFORMATION FOR SEQ ID NO:6:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- 20 (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GACGCTGGY TTYTIGCIAA A 21

- (2) INFORMATION FOR SEQ ID NO:7:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

30 GACGCTGGY TTYTIGCIAA G 21

- (2) INFORMATION FOR SEQ ID NO:8:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 5
- (B) TYPE: amino acid
- 35 (C) STRANDEDNESS: single
- (D) TOPOLOGY: helix
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

40 Gly Gln Leu Asp Trp  
1 5

- (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCAAGTGTAC CARTCNAGYT GNCC 24

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

15

CCAAGTGTAC CARTCRTAYT GNCC 24

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TAGTCGACTG GCTTYTGGCC AAAiictGGG TSCG 34

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

30

GCGGATCCCT CCACCAGGNY TGSAGYTC 28

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24
- (B) TYPE: nucleic acid

35

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGTATCTACT ACCCAAGTGA TGAG 24

5 (2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TACCCAAGTG ATGAGTCTGT C 21

15 (2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

20 GAAGACCTCA GGCAGCAGAT GTG 23

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TCATGGAAGG AGAACTCGGC AT 22

30 (2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ACGGATCCAG CATGCCGAG TTCAGGGTCA G 31

5 (i) (2) INFORMATION FOR SEQ ID NO:18:  
SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

10 CGGAATTCAT GTCATCTGGG CCTGTGTTC 30

15 (i) (2) INFORMATION FOR SEQ ID NO:19:  
SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGCCCTCTCGC CATCCAGCT 19

20 (i) (2) INFORMATION FOR SEQ ID NO:20:  
SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TGTTCCCTG GGATTAGAT GGA 23

30 (i) (2) INFORMATION FOR SEQ ID NO:21:  
SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGTATCTACT ACCCAAGTGA TGAG 24

5 (2) INFORMATION FOR SEQ ID NO:22:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TGGGATGTCA TCTGGGCTG T 21

10 (2) INFORMATION FOR SEQ ID NO:23:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
15 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AACTCACCCC CACCACCATA CACA 24

20 (2) INFORMATION FOR SEQ ID NO:24:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

25 TTCCCGCCTC CATCTCCCAA AGT 23

30 (2) INFORMATION FOR SEQ ID NO:25:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 662  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

MGLYRIRVSTGASLYAGSNHQQVLWLVGQHGEAALGKRLWPARGKETLKVEVPEYLGPLL FVKLRKRHLLKDDAWFCNW

5 ISVQGGAGDEVRFPCYRWVEGNVLSLPEGTGRTVGEDPQGLFQKHREEELEERRKLYRWGNWKGDLILNMAGAKLYDL  
PVDERFLEDKRVDFEVS LAKGLADLA IKDSL NVLT C WKD LDDFNRI FWCGQSKLAERVRDSWKEDALFGYQFLNGANPVV  
LRRSAHLPARLVFPFGMEELQAQLEKELEGGLFEADFSLLDGIKANVILCSQQHLAAPLVMLKLQPDGKLLPMVIQLQL  
PRTGSPPPPLFLPTDPPMAWLLAKCWRSSDFQLHELQSHLLRGHLM AEVIVVATMRCLPSIHPIFKLIIPHLYTLEIN  
VRARTGLVSDMGIFDQIMSTGGGGHVQLLKQAGAFITYSSFCPPDDLADRGLLGVKSSFYAQDALRLWEIYRYVEGIVS  
LHYKTDVAVKDDPELQWCREITEIGLQGAQDRGFPVSLQARDQVCHFVTMCIFTCTGQHASVHLGGQLDWYSWVPNAPCT  
MRLPPPTTKDATLETVMATLPNFHQASLQMSITWQLGRRQPMVAVGQHEEYFSGPEPKAVLKKFREELAALDKIEIR  
NAKLDMPEYELRPSVVENSVAI

- 10 (i) (2) INFORMATION FOR SEQ ID NO:26:  
SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown  
15 (ii) MOLECULE TYPE: peptide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Glu Leu Gln Xaa Trp Trp Tyr  
1 5

- 20 (i) (2) INFORMATION FOR SEQ ID NO:27:  
SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown  
25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Asp Val Trp Leu Leu Ala Lys  
1 5

- 30 (i) (2) INFORMATION FOR SEQ ID NO:28:  
SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

35 Gly Gln Phe Asp Ser  
1 5

- (i) (2) INFORMATION FOR SEQ ID NO:29:  
SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCAAGCGCAS SARTCRAAYT GNCC 24

- (2) INFORMATION FOR SEQ ID NO:30:
- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GAGCTTTGTC TCTGAAATAG TCAG 24

- (2) INFORMATION FOR SEQ ID NO:31:
- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

15

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GTGAGGAATC AATAGCTTGA AGAG 24

- (2) INFORMATION FOR SEQ ID NO:32:
- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

30 GATGTGTGAC AGCCTCATGG ATG 23

- (2) INFORMATION FOR SEQ ID NO:33:
- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 10
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CAAGCTTAGG AGGATGGCGA AATGCAGG 28

(2) INFORMATION FOR SEQ ID NO:34:  
(i) SEQUENCE CHARACTERISTICS:  
5 (A) LENGTH: 20  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

10 GGAATTCATG TTAGATGGAG AACTGTT 28

(2) INFORMATION FOR SEQ ID NO:35:  
(i) SEQUENCE CHARACTERISTICS:  
15 (A) LENGTH: 10  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Gly Gln Tyr Asp Trp  
1 5

(2) INFORMATION FOR SEQ ID NO:36:  
(i) SEQUENCE CHARACTERISTICS:  
20 (A) LENGTH: 10  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
25 (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Xaa Val Asp Trp Leu Leu Ala Ala Lys Xaa Trp Val Arg  
1 5 10

30 It will be understood that various details of the  
invention may be changed without departing from the scope  
of the invention. Furthermore, the foregoing description  
is for the purpose of illustration only, and not for the

purpose of limitation--the invention being defined by the claims.